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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

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OBITUARY NOTICE

VICTOR ALEXANDER HERBERT HUIA ONSLOW.

RARELY indeed in the history of Science can significant researches have been accomplished under conditions calling for such heroic courage as that displayed by the subject of this brief memoir. In August 1911 while still a student at Cambridge he was climbing in the Tyrol with Charles Meade the Himalayan explorer. He dived into a mountain lake, striking his head on a submerged rock. This so damaged the spine that the spinal cord was practically severed at the level of the sixth cervical vertebra, and paralysis from the waist downwards, with incomplete use of the hands and arms, was the necessary consequence.

Though never again to move from his bed Onslow faced his tragic destiny with high courage. He determined still to be of use in the world, and the moment the verdict of "incurable" was pronounced he settled down to pursuits involving not only intense intellectual activity but also scientific experimental work most of which, in spite of his grave disabilities, he carried out by himself. To this end he with immense determination compelled his partially paralysed arms and hands to the attainment of quite remarkable skill. No one ever stood at his bedside without feeling almost overwhelmed with the poignancy of what he saw. On a raised pillow the handsome intellectual face lit up with keen interest; the powerless body....

Huia Onslow, younger son of the fourth Earl of Onslow, was born on Nov. 13, 1890, at Government House, Wellington, New Zealand, while his father was Governor. He was the godson of Queen Victoria. The circumstances of his infancy were romantic. When about 12 months old he was inducted as Chief of the Ngathuia, an ancient and powerful Maori tribe. His unique Christian name, Huia, was given him in token of friendship between the British Government and the Maori race, and has reference to the Huia bird of New Zealand, of which the feathers were the tribal emblem of the Ngathuia! When fourteen years of age he again visited New Zealand and was welcomed with high festival by his tribe.

He was educated at Eton and Trinity College, Cambridge. While at the University he read natural science for one year, and then for two years turned his attention to mechanical science with the intention of qualifying for the Parliamentary Bar where he expected to find a knowledge of engineering very useful. He entered at one of the Inns of Court in 1911, but almost immediately after this, and only two months after he had qualified for his Cambridge degree, occurred the tragedy which cut him off from an active career. Active that is in the common sense; as already said there was scarcely a breach in his real

activities. During the year which followed that of his accident he read intensely, as a real student and not for pastime, the literature of a wide range of subjects—philosophy, psychology, genetics—taking the keenest interest in literary matters, in Art, and in current affairs. In this year while living in London he did experimental work in psychology, and started the Mendelian Studies upon mice and rabbits which were to continue for the rest of his life.

In the next year he started definite microscopical and chemical work on hair structure and hair pigments, a line of enquiry which always interested him, in the first instance because of its relation with his studies in genetics. At this time, as later, he used the microscope himself, and executed nearly all the manipulations connected with the instrument which stood on a little platform stretched over his bed. The chemical work was partly, but by no means exclusively, done by an assistant under his close and continuous supervision. At this time, too, he was writing much; contributing various literary articles and poems to the *Spectator* and other high class Journals.

When the war arrived it was characteristic of Onslow that he should turn his mind to possible service. He worked, not without real success, at methods for the detection of gun-fire by daylight, and also upon sound ranging. He became Joint Secretary of the Soldiers' and Sailors' Families Association for the North-western district of London. He did not however give up his scientific work at this time. He studied with S. W. Cole some of the properties of urobilin and allied pigments, and, in particular, worked at the causes of dominant and recessive whiteness in animals.

In 1915 he returned to live permanently at Cambridge and fitted up at his house a laboratory into which his couch could be wheeled. In this year a paper appeared in the *Proc. Roy. Soc.* in which he showed that Recessive Whiteness (Albinism) in rabbits is due to the absence of a tyrosinase; Dominant Whiteness on the other hand was shown to be due to the presence of an inhibitory factor. A little later there appeared in this Journal a paper on the production of the black markings on the wings of *Pieris*. Mendelian experiments with Lepidoptera were now started and continued throughout his life. Many important and beautiful results were obtained which are described in a series of papers in the *Journal of Genetics*. He worked further with S. W. Cole on the metabolism of bacteria.

About 1917 he became interested in protein chemistry and made himself an expert dealer with amino acids. Onslow was particularly anxious to solve the elusive problem presented by the quantitative separation of tryptophane and was near to its solution at the time of his death. The results of this investigation though not quite final will be shortly published. Work which is remarkable in itself, but exceptionally remarkable because of the untoward circumstances under which the skill requisite for its accomplishment was attained, is described in the paper "On a Periodic Structure in many Insect Scales and the Cause of their Iridescent Colours" which appeared in the *Philosophical Transactions* for 1921.

It is impossible in a brief notice to do full justice to his scientific work or to his other activities. The latter indeed were diverse enough to the end. He was for instance an authority on gems, and on jewellery, which he designed, making his own drawings and paintings.

In 1919 he took a step which added greatly to the happiness of his remaining years. He had indeed the gift of exacting happiness from his so grudging destiny. He married Muriel Wheldale; one whose scientific work is very familiar to the readers of this Journal.

By a pleasant and acceptable fiction Onslow claimed to be a member of the Biochemical Department at Cambridge. It became less a fiction after his marriage, for his wife brought his thoughts to us, and reported our activities to him. Indeed, after all, it was no fiction. We knew always that a short way off a colleague was daily conquering difficulties compared with which our own could be as nothing. How could we fail to work the better for that knowledge? Onslow died June 27, 1922. The tale of his years was—thirty-one!

F. G. H.

PAPERS PUBLISHED.

1. "The French Commission on Depopulation." *Eugenics Review*, 1913.
2. "Depopulation and Eugenics." *Eugenics Review*, 1913 and 1914.
3. "Hairs and Hair Pigments." *Knowledge*, 1914.
4. "Some White Ruthenian Folk-Songs." 2 parts, with Miss Iwanowska. *Folk-lore*, 1914.
5. "A Contribution to our Knowledge of the Chemistry of Coat Colour in Animals and of Dominant and Recessive Whiteness." *Proc. Roy. Soc.* 1915.
6. "On the Formation of Hair Pigment." *Knowledge*, 1915.
7. "The Cause of Albinism and Dominant Whiteness." *Knowledge*, 1915.
8. "On the Development of the Black markings on the Wings of *Pieris brassicae*." *Biochem. J.* 1916.
9. "On a substitute for Peptone and a Standard Nutrient Medium for Bacteriological Purposes," with S. W. Cole. *Lancet*, 1915.
10. "On a rapid Method for the cultural Differentiation of the Typhoid and Paratyphoid Bacilli A and B," with S. W. Cole. *Lancet*, 1916.
11. "A Note on certain names recently applied to sable mice." *Journal of Genetics*, 1917.
12. "Note on the nature of the Growths in Colloidal silica solutions." *Proc. Roy. Soc.* 1918.
13. "The Inheritance of Wing Colour in Lepidoptera. I. *Abraxas Grossulariata* var. *lutea*." *J. Genetics*, 1919.
14. II. "Melanism in *Tephrosia consonaria*." *J. Genetics*, 1919.
15. III. "Melanism in *Boarmia consortaria*." *J. Genetics*, 1920.
16. IV. "Melanism in *Boarmia abietaria*." *J. Genetics*, 1920.
17. V. "Melanism in *Abraxas grossulariata*." *J. Genetics*, 1921.
18. VI. "*Diaphora mendica* and var. *rustica*." *J. Genetics*, 1921.
19. VII. "Melanism in *Hemerophila abruptaria*, var. *fuscata*." *J. Genetics*, 1921.
20. "On a Periodic Structure in many Insect Scales, and the Cause of their Iridescent Colours." *Trans. Roy. Soc.* 1921.
- 20 (a). "The Iridescent Colours of Insects." *Nature*, 1920.
21. "On the Stability of Tryptophan in Baryta Hydrolysis." *Biochem. J.* 1921.
22. "On the Nature of the Substances precipitated by Mercuric sulphate from Hydrolysed Caseinogen, with special reference to the Estimation and Isolation of Tryptophan." *Biochem. J.* 1921.
23. "A Note on the Inheritance of Steel Coat-Colour in Rabbits." *J. Genetics*, 1922.

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1. "How much should we eat?" *Land and Water*, 1917.
2. "The Colour of Butterflies." *Country Life*, 1920.
3. "Hidden Dangers in Diet." *World's Work*, 1919.
4. "Symbiosis: the Art of Living together." *World's Work*, 1920.
5. "Structure of Butterflies' scales." *Conquest*, 1920.
6. "The Microbe and its Ways." *Conquest*, 1921.
7. "Life on the Roof of the World, the Problems of the Mount Everest Expedition." *Conquest*, 1921.
8. "Black Moths." *Conquest*, 1921.
9. "The Butterfly and the Ant." *Conquest*, 1922.
10. "Oriental and cultured Pearls." *Conquest*, 1922.
11. "Science and efficiency." *Conquest*, 1922.
12. "Fair and Dark. Is there a predominant type?" *Eugenics Review*, 1920.

Papers still to be published:

Dalmatians.

Tryptophane.

Inheritance of wing-colour in Burnet and Scarlet tiger.

Inheritance of wing-colour in *Abraaxas*, var. *exquisita*.

Biochemistry of Lymph of larvae.

Popular. "Clean Milk" and "Perfumes."

I. SOME NEW REACTIONS FOR THE DETECTION OF STEROLS.

By GEORGE STAFFORD WHITBY.

From the Department of Chemistry, McGill University, Montreal.

(Received November 16th, 1922.)

OBSERVATIONS made in relation to the colour reactions in vogue for the detection of sterols have indicated that in their general character all or most of these reactions are in essence similar, and have led to the discovery of several new reactions which present certain interesting features including a greater degree of delicacy than that possessed by reactions now employed. In what follows, first three new reactions for sterols and a reaction for sterolins are described, and then some considerations are brought forward concerning the mechanism of the colour reactions shown by sterols.

Reaction A. To 2 cc. of a chloroform solution of the sterol (containing preferably 1-2 mg. of the sterol) are added 2 cc. of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportions 50 vols. : 1 vol., and the contents of the test-tube are then shaken. When the layers have separated, the upper, chloroform layer is seen to be cherry-red in colour, and the lower, sulphuric acid layer to be brownish-red in colour and to show an intense green fluorescence. The upper layer is then poured off into a dry test-tube and treated with two or three drops of acetic anhydride; as a result it assumes a bright blue colour, which lasts for a considerable time, but passes into a green within, say, an hour.

The reaction is much more sensitive and striking than the Salkowski [1872] reaction, of which it may be regarded as an elaboration. In the Salkowski reaction, a chloroform solution of a sterol is shaken with an equal volume of conc. sulphuric acid; when the liquids have separated, the chloroform layer is seen to be brownish-yellow, and the sulphuric acid layer to be yellow-brown with a green fluorescence; on allowing the test-tube to stand for several hours, the sulphuric acid layer becomes deeper and redder in colour and more strongly fluorescent, while the chloroform layer assumes, if the sterol was present in sufficient amount, a cherry-red or purple colour. Reaction A described above has several advantages over the just-described Salkowski reaction, namely: (a) the cherry-red colour is obtained immediately in the former, whereas in the latter it is necessary to wait several hours for its appearance; (b) the former reaction is given by a much smaller quantity of the sterol than the latter (*vide infra*); (c) the coloured chloroform solution obtained in the

former is converted by acetic anhydride into a comparatively lasting blue, whereas when the cherry-red solution obtained in the latter is treated with the same reagent a blue stage, if recognisable, is very transient, and does not add a well-defined feature to the reaction.

A comparison of the degree of delicacy of Reaction A and the Salkowski reaction showed that the former is more than ten times as sensitive as the latter.

2 cc. of cholesterol solution were used in each of the tests made in this connection. When 0.01 mg., *i.e.* 1 part in 100,000 parts, of cholesterol was present, in Reaction A a faint fluorescence could be seen in the sulphuric acid layer on allowing the contents of the test-tube to stand or on warming them, although no colour could be seen in the chloroform layer. This amount of sterol represents approximately the limit at which Reaction A gives any result. The Salkowski reaction gave no result with this amount. When 0.1 mg. sterol was present, Reaction A gave a comparatively deep reddish-brown colour and a very marked fluorescence in the sulphuric acid layer, and, if the contents of the test-tube were heated, a barely discernible purple colour in the chloroform. When the same amount of sterol was present, the Salkowski reaction gave no colour in the chloroform and no colour or fluorescence in the sulphuric, although a faint colour and fluorescence developed in the latter if the contents of the tube were heated and then allowed to stand for about ten minutes. Even when 1 mg. of sterol was present, a cherry-red colour in the chloroform was not usually noticeable in the Salkowski reaction; any faint colour which might possibly have developed during the night having disappeared by the morning. With 1 mg. of sterol, Reaction A gave an immediate cherry-red colour in the chloroform.

Reaction A was applied with positive and essentially identical results to three samples of cholesterol and five samples of phytosterol¹. In this reaction, amyirin gives in the chloroform no colour but in the sulphuric acid an orange-red colour (which soon becomes blood-red) and a deep green fluorescence; abietic acid gives nothing very characteristic: the chloroform becomes merely pale brown and the sulphuric acid dark red-brown and slightly fluorescent.

A modification of the Salkowski reaction, made by Hesse [1881], and consisting in using sulphuric acid of specific gravity 1.76 instead of 1.84, has the advantage over the Salkowski reaction of giving a characteristic colour (rose) in the chloroform immediately, but it has the disadvantage of not giving fluorescence in the acid and of being less sensitive than the ordinary Salkowski reaction. It must be considered as less generally useful than either the Salkowski reaction or Reaction A.

¹ These samples were as follows: (a) a sterol from the resin of *Hevea* rubber [Whitby and Dolid, 1921]; (b) phytosterols from *Adonis vernalis*; (c) sitosterol from *Echinacea* root; (d) and (e) preparations of sitosterol from wheat. The author is much indebted to Dr F. W. Heyl, the Upjohn Co., Kalamazoo, Mich. for samples (b) and (c), and to Prof. L. Kahlenberg, University of Wisconsin, for samples (d) and (e).

Reaction B. To 2 cc. of a solution of sterol in glacial acetic acid, containing conveniently 0.2–0.5 mg., are added with shaking 25 drops of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportion 50 vols. : 1 vol. The result is a rose-coloured and fluorescent solution.

The rose colour, which has a tinge of purple, is not permanent, but on standing changes to yellow-brown. When only 0.01 mg. of sterol is present, the rose colour changes quickly, but more usually (*i.e.* with an amount of sterol of the order first mentioned) no change in the full, initial brightness of the colour is apparent for about two minutes, and the change to brown is not complete until an hour or more has elapsed.

This reaction is more sensitive than any colour reaction hitherto proposed for the detection of sterols. It is approximately twice as sensitive as the Liebermann-Burchard reaction [Liebermann, 1885; Burchard, 1889], although, unlike the latter, it is not suited to the quantitative determination of sterols. In Reaction B colour is just recognisable with 0.005 mg. of sterol per cc., *i.e.* the limit of sensitiveness of the reaction is 1 in 200,000. With 1 mg. per cc. the colour developed is too deep to allow one to see through the solution in a test-tube. A suitable concentration at which to apply the reaction is 0.1–0.25 mg. sterol per cc.

The reaction may be applied to an ethereal instead of an acetic acid solution of a sterol; but in this connection it should be noted that on standing for several hours a blank test will show a brown colour.

In Reaction B amyirin gives a cherry-red, strongly fluorescent solution, which becomes brown on long standing; abietic acid, a not very characteristic green-brown colour¹.

Reaction C. A few milligrams of a sterol are added to one drop of acetic anhydride on a piece of porcelain and gently heated until it has melted and excess of anhydride has been driven off. The melt is allowed to cool completely, and is then moistened with conc. nitric acid. Within a few seconds the material assumes a blue or blue-green colour.

This reaction is chiefly of value for cholesterol; it is of less value for phytosterols. Five samples of phytosterol² to which the reaction was applied behaved somewhat differently from cholesterol: even when the reaction was carried out with great care (the melt being distributed in as thin a layer as possible), they gave a much less intense colour than did cholesterol. The difference in behaviour of cholesterol and phytosterols in this reaction is not, however, sufficiently sharp to allow of the reactions being used as a means of distinguishing animal and vegetable sterols.

¹ The behaviour of amyirin and abietic acid in certain other sterol colour reactions may be put on record here. In Tschugajeff's reaction (see later) amyirin gives a red, which is somewhat browner than that given by a sterol and is unaccompanied by fluorescence; abietic acid gives a still browner red which is, however, accompanied by fluorescence. In Lifschütz's reaction (see later) neither substance gives a characteristic colour but merely a pale brown colour.

² Described in footnote, p. 6.

The author could not confirm the statement of Kahlenberg [1922] that arsenic chloride provides a means of distinguishing cholesterol from phyto-sterols by yielding a cherry-red solution with the former and a colourless solution with the latter. Each of the five samples of phytosterol referred to above gave a cherry-red solution in arsenic chloride.

A fusion test for the detection of cholesterol has been described by Obermüller [1891], but does not appear to be very useful, as it would seem to demand exceptionally dry samples of cholesterol for success. The present author obtained negative results on applying Obermüller's reaction to samples of cholesterol taken directly from stock bottles.

Sterolin reaction. Sterolins¹ give most of the colour reactions of sterols (*vide infra*). The following reaction, which depends in part on the sterol portion and in part on the glucose portion of the molecule, has been designed in conjunction with Mr J. Dolid, for recognising sterolins and at the same time distinguishing between sterolins and sterols.

1-2 cc. of conc. sulphuric acid is poured on to a few particles of a sterolin contained in a test-tube, and the mixture is warmed gently until the solid has gone into solution; the liquid is then cooled, and a cold, saturated, aqueous solution of thymol is poured on top of it. The result is an orange colour and a strong green fluorescence in the lower layer and a violet ring at the junction of the upper and lower layers. On allowing the test-tube to stand, the violet colour tends to spread through the upper layer.

When the reaction is applied to a sterol, a lower layer similar to that described above is obtained, but a violet ring is missing.

The sample of sterolin to which the above reaction was applied was isolated from the resin of *Hevea* rubber [Whitby and Dolid, 1921]. Its behaviour in the various sterol colour reactions was as follows. It gave Reaction B, the Liebermann-Burchard, the Tschugajeff, and the Lifschütz reactions. In Reaction A its behaviour was slightly different from that of a sterol, due doubtless to the circumstance that it is only very slightly soluble in chloroform: fluorescence occurred in the sulphuric acid layer, as in the case of a sterol; the colour in the chloroform layer was not, however, as in the case of a sterol, cherry-red, but a brownish yellow, which soon deepened to a red-brown; and, when the chloroform layer was poured off and treated with acetic anhydride, it became blue, but the blue remained for a shorter time than in the case of a sterol.

A consideration of the colour reactions applicable to the detection of sterols in solution indicates the existence of a general similarity between these superficially different reactions. In all or nearly all the reactions in question three points in common are discernible, namely, (a) the dehydration of the sterol

¹ The name phytosterolins has been given by Power and Salway [1913] to glucosides of phytosterols, such as have been recognised during recent years in a considerable number of plants.

molecule with the production of a colourless substance, (b) the appearance of a coloured product by the interaction of this first substance with a second (coupling) substance derived from the sterol or introduced in carrying out the reaction, (c) the use of an agent for rendering the medium anhydrous. As is pointed out below, the first substance is probably a hydrocarbon—*e.g.* a cholesterylene or cholesterylin—formed by the withdrawal of the elements of water from the sterol. The dehydrating agent responsible for its production may or may not also be the agent used to render the medium anhydrous. The coloured products obtained in the sterol colour reactions are very sensitive to traces of moisture. The actual colour obtained depends upon both the thoroughness with which the medium is dehydrated and the nature of the second (coupling) substance. These points can be illustrated by considering some of the individual reactions.

The Salkowski reaction. If the cherry-red chloroform layer is poured into another test-tube, it is usually seen to lose its colour as a result of the transference. That the destruction of the colour is due simply to the small amount of moisture present in a nominally dry test-tube is shown by, among other observations, the fact that the colour can be restored, not only by conc. sulphuric acid (as was noticed by Salkowski), but also by phosphorus pent-oxide.

In the Salkowski reaction the sulphuric acid acts both to produce the first, colourless substance by its action on the sterol and to render the chloroform anhydrous. The presence, immediately after the introduction of the sulphuric acid, of the first, colourless substance, capable, by reacting with a second substance, of giving a coloured product, is shown by the observation that, if the chloroform layer is poured off at once, before a cherry-red colour has developed, and is then treated with formaldehyde¹, which serves as the coupling substance (cf. Reaction A, *infra*), a cherry-red or purple colour is obtained at once. In the ordinary Salkowski reaction a second (coupling) substance apparently arises only slowly. The rate at which the second substance necessary for the appearance of colour arises seems to be different in different solvents, and is, for example, noticeably slower when chlorobenzene is used as a solvent for the sterol than when chloroform is used.

Reaction A. In the reaction the first substance couples at once with formaldehyde to give a cherry-red product. Unlike the paler cherry-red colour produced in the ordinary Salkowski reaction, the colour produced in Reaction A is not destroyed by pouring the chloroform layer into another test-tube. Acetic anhydride changes the colour through purple and then through blue to green. The characteristic feature here is the comparative persistence of the blue stage, which has hitherto been discernible in sterol colour reactions (*e.g.* in the Liebermann-Burchard reaction) only as a very fleeting stage. By choosing a suitable solvent, in which the blue colouring matter is insoluble, the latter can be actually isolated as a solid.

¹ In, say, chloroform or ethereal solution.

Two drops of conc. sulphuric acid were added to 3 cc. of a solution of cholesterol in ethyl bromide. The solution immediately took on an orange colour (which became blood-red within a few minutes) and appeared fluorescent. A small amount of a brown solid separated in flocks. The blood-red solution was treated with a few drops of acetic anhydride, and then allowed to stand for half-an-hour. While standing, the solution passed through a purple stage, then became blue, and gradually deposited a blue solid. That this solid represents the blue product seen in solution in various of the sterol reactions, is indicated by the fact that it was found to be soluble in chloroform, and, when dissolved in the latter, to suffer a change of colour to green on treatment with acetic anhydride.

Reaction B. Here, as in the previous reaction, sulphuric acid both dehydrates the medium and acts on the sterol to produce the first, colourless substance, and the latter then reacts with formaldehyde to give a coloured product. This reaction is more sensitive than Reaction A because of the miscibility of the sulphuric acid with the solvent. In the case of the Salkowski reaction and Reaction A some of the colour-producing substance goes into the sulphuric acid layer. In this connection the following observations may be noted.

(1) That, alike in the Salkowski reaction and in Reaction A, the same substance is in question in the two layers, is indicated by the observation that formaldehyde, which, as already mentioned, intensifies the colour in the chloroform, also intensifies the depth of the colour and the fluorescence in the sulphuric acid.

(2) If in Reaction A the two layers are shaken together vigorously, the upper layer can be rendered almost colourless, owing to the transference of colour to the sulphuric acid layer.

(3) If conc. sulphuric acid is dropped into a solution of cholesterol in carbon tetrachloride, the latter assumes at once, as the acid drops through it, a deep yellow colour, and the sulphuric acid forms a colourless layer at the bottom; if now the two layers are shaken together well, the colour goes almost completely into the sulphuric acid layer, the upper layer becoming nearly colourless.

In connection with carbon tetrachloride, it is interesting to note that the purple-coloured product obtained in various of the sterol colour reactions is insoluble in this solvent and can be isolated by its use.

(a) The above-mentioned yellow, carbon tetrachloride layer was poured off and treated with acetic anhydride. A dark-purple solid gradually separated, the liquid itself finally becoming green. The solid was removed, and was found to be soluble in warm chloroform. The purple chloroform solution of the solid, when treated with acetic anhydride, became converted through blue to green.

(b) The yellow carbon tetrachloride solution was treated with formaldehyde. There separated a purple-coloured solid with properties similar to those of the purple solid just mentioned.

It thus appears that the purple solid, mentioned under (a), and obtained by treating the first, colourless substance in carbon tetrachloride with acetic anhydride, is essentially identical with the purple or cherry-red substance obtained by treating the first colourless substance with formaldehyde.

Liebermann-Burchard reaction. In this reaction [Liebermann, 1885; Burchard, 1889] a green colour is produced by the addition of a drop of conc. sulphuric acid to a solution of a sterol in acetic acid containing some acetic anhydride. The sulphuric acid produces the first, colourless substance from the sterol, and the acetic anhydride then reacts with this substance to give a purple-coloured product, which it then changes to a blue and finally to a green product. The acetic anhydride also serves to dehydrate the medium. The power of acetic anhydride to change the colour of the earlier coloured stages and at the same time to dehydrate the medium, is shown in the following observations.

(a) The cherry-red chloroform layer obtained in Reaction A was poured off and diluted with chloroform from a stock bottle until it became colourless. (The amount of moisture in an ordinary sample of chloroform is sufficient to discharge the colour.) Acetic anhydride was then added. The result was to produce a blue colour (which later turned green), just as it would have been had the colour not first been discharged.

(b) To a blue chloroform solution obtained in Reaction A drops of water were added until the colour was discharged. (The colour could be restored by adding phosphorus pentoxide.) A little acetic anhydride was then added. The result was a green colour.

Tschugajeff reaction. In this reaction [Tschugajeff, 1900] an eosin-red colour is obtained by boiling an acetic acid solution of a sterol with zinc chloride and acetyl chloride¹. The zinc chloride acts both to withdraw water from the sterol molecule and to dehydrate the medium. In the latter function it is doubtless assisted by the acid chloride.

It was found that if an acetic acid solution of a sterol is boiled with powdered, dry zinc chloride, without the addition of an acid chloride, a purple colour develops. Hence it would appear that zinc chloride alone is capable of producing from a sterol both a first and a second substance, which together give rise to what has previously been referred to as the first coloured stage (cherry-red, purple, or rose). As in the case of the Salkowski reaction, the amount of the second substance formed is apparently insufficient to lead to the full development of colour which the amount of the first colourless substance produced is capable of yielding; for, if a little formaldehyde is introduced (as vapour) into the solution, the purple colour obtained on boiling is deepened.

Lifschütz reaction. In this reaction [Lifschütz, 1898] benzoyl peroxide serves to dehydrate the medium and to react with the first, colourless substance, formed from the sterol by the action of sulphuric acid.

It would appear that the hydrocarbons—*e.g.* cholesterylenes and cholesterylins—which can be obtained from sterols by the action of sulphuric acid

¹ It was found that the acetyl chloride may, with identical results, be replaced by benzoyl chloride.

or zinc chloride, and have been studied by Zwenger [1848, 1849] and by Mauthner and Suida [1896], are concerned in the reactions discussed above.

A mixture of hydrocarbons, prepared from cholesterol according to the procedure of these authors, was dissolved in chloroform. The solution thus obtained was colourless, but when warmed with phosphorus pentoxide became rose- or cherry-red. If a little formaldehyde vapour was passed into the solution before the addition of the pentoxide, a deeper purple colour was obtained. If the purple solution was poured off and treated with acetic anhydride, its colour changed through blue to green. These results are strictly parallel with those observed in connection with the sterol colour reactions.

It appears that in the mixture of hydrocarbons (? with possibly other substances) which sulphuric acid produces from cholesterol there are present both the substances previously referred to as the first, colourless substance and the second (coupling) substance; as it is sufficient to dissolve the mixture in a thoroughly dehydrated medium for a cherry-red colour to make its appearance. It appears, further, that, as was seen to be the case in the Salkowski and Tschugajeff reactions, here too an insufficient amount of the second (coupling) substance is present; as the introduction of formaldehyde deepens the colour obtained.

SUMMARY.

Three new colour reactions (A, B and C) for the detection of sterols are described. Reaction A is more striking and sensitive than the usual chloroform-sulphuric acid reaction. Reaction B is more sensitive than any sterol colour reaction hitherto described.

A reaction is described for detecting sterolins and distinguishing them from sterols.

In a general discussion of the reactions applicable to the detection of sterols in solution it is concluded that all or almost all such reactions are, in their essential features, similar.

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II. STUDIES IN EXPERIMENTAL TETANY.

I. ON THE DISTRIBUTION OF CALCIUM IN THE PLASMA AND CELLS.

II. ON THE VARIATIONS IN COLLOIDAL AND IONIC CALCIUM.

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I.

THEORIES PUT FORWARD WITH REGARD TO THE CAUSATION OF TETANY.

ALTHOUGH much research work has been done and a great deal written on this subject, it may not be out of place here briefly to recapitulate the main theories which have been put forward in an attempt to explain the chemical changes occurring in the blood and their relationship to the symptoms following a loss of function of the parathyroid glands.

The first hypothesis formulated was that the condition was due to a disturbance of the metabolism of calcium salts whereby the tissues and body fluids were deprived of soluble calcium, hence the irritability of the nervous system. This was based upon work of MacCallum [1902-1904] and Loeb [1902, 1, 2; see also 1915] in 1902 and upon researches by Sabbatani who showed that salts of bivalent metals have a depressing effect on the central nervous system, and that such salts counteract the effect of calcium-precipitating substances on the cerebral cortex.

Further confirmation of this theory was forthcoming in the work by Quest [1906] upon the calcium content of cerebral tissue in tetany and by the demonstration of the curative effect of calcium salts upon tetany by MacCallum and Voegtlin [1909]. These workers had previously clearly shown that tetany was associated with a deficiency of calcium salts. That the injection of calcium salts is efficacious in the treatment of tetany in children has been more recently confirmed by Howland and Marriott [1916].

In 1915 Wilson, Stearns and Thurlow [1915] brought forward evidence to the effect that after parathyroidectomy a condition of alkalosis may develop, which condition is neutralised by acid products formed by the muscular activity associated with the muscle tremors incident to tetany.

That the condition preceding an acute attack of tetany is one of alkalosis, they deduced from a study of the variations in the values of the dissociation constant of oxyhaemoglobin, the alveolar carbon dioxide pressure, and the

hydrogen-ion concentration of the blood of dogs subjected to parathyroidectomy. They further showed that the condition of acidosis following several attacks of tetany and the injection of a mineral acid are associated with relief from tetany. In this condition of acidosis the dissociation constant of oxyhaemoglobin is lowered as is also the alveolar carbon dioxide pressure.

Wilson, Stearns and Janney [1915] also indicate that there is a sudden diminution in the excretion of acids and ammonia and a decrease in the ammonia ratio and the hydrogen-ion concentration of the urine immediately following parathyroidectomy. With the onset of tetany there is an increased elimination of acids and ammonia with an increase in the hydrogen-ion concentration value of the urine.

The third hypothesis which has been brought forward is that the condition of tetany is due to the circulation of some toxin or toxins produced by the breakdown of body protein and to be found in the blood and urine of the patient. This theory was the outcome of work by Noël Paton, Findlay and Burns [1915, 1916] and was first published in 1915.

The idea that some product of muscle protein decomposition was the responsible factor was not then entirely new, it having been suggested by Pekelharing and Van Hoogenhuyze [1910] some five years previous to the publication of the researches of Paton and his co-workers.

It is well known that the injection of guanidine sulphate or methylguanidine [Paton, Findlay and Burns, 1915] gives rise to symptoms of hyper-irritability of nerves and it is to Koch [1912] that the credit is due for first discovering the presence of methylguanidine in the urine of dogs suffering from parathyroid tetany.

THE DISTRIBUTION OF CALCIUM IN THE PLASMA AND CELLS.

Numerous estimations upon the total calcium of blood, of plasma and of serum have been carried out, and while a certain divergence of results exists, the general average of results arrived at by various workers is practically the same. In their earlier work MacCallum and Voegtlin stated that the per cent. of calcium in whole normal blood was about 13.3 mg. while in tetany blood it was about 5.4 mg. Several years later these workers by means of a different analytical method arrived at the conclusion that their former results were too high and that more correct figures for the percentage of calcium in normal and tetany blood, would be 6.1 mg. and 2.7 mg. respectively.

Variations in results are undoubtedly due to difference in the methods used for the estimation of blood calcium. Of the two methods more generally used in recent years, namely those of Lyman [1917] and Halverson and Bergheim [1917], it seems that the experimental error is much more under control with the former than with the latter. A nephelometric method which depends upon the accurate matching of shades or depth of colour, always introduces a personal error, and such a method cannot safely be adopted until by practice he who uses it is assured by stringent tests that his readings

are within 0.5 to 1 mm. of the correct figure. The latter method is one of titration with potassium permanganate, a titration in which the end point is rather difficult of determination and one in which one drop of the permanganate solution too many or too few may result in an error of $\pm 5\%$.

In the present investigation Lyman's method has been used and has proved very satisfactory for estimating small amounts of calcium. The nephelometer used was made by taking a Duboseq colorimeter, and replacing the cups and plungers with hard rubber cylinders into which short prepared test-tubes fitted accurately. The test-tubes were supported by means of their flanged edges resting in two lateral slits cut into a hard rubber plate which was screwed on to the colorimeter beneath the prism. The cylinders, supported by movable rings resting on the cup supports, were so adjusted that when the tops were in contact with the hard rubber plate, thereby excluding all light, the indicators on the millimeter scale stood at the zero mark.

The nephelometer-colorimeter attachment or illumination box was made of wood painted black on the outside and white within, the partition placed between the lamps: the colorimeter contained a plate of frosted glass, and the illumination consisted of one set of two 100 c.p. 110 volt lamps and one set of two 100 c.p. 220 volt lamps, so arranged that any number could be used for illumination.

In determining the distribution of calcium in plasma and cells, it was necessary to obtain accurate values for the proportion of cells to plasma in whole blood. For this purpose it was found that the ordinary haematocrit was not satisfactory and the following method was adopted. A piece of pressure glass tubing 10 cm. long having an internal diameter of 1 mm. and a wall thickness of 3 mm. was taken and the ends made smooth with a file.

To a 50 cc. metal centrifuge holder a small right-angled piece of iron was attached so that it formed an inverted L, the arm of the letter being placed over the mouth of the cup and having a tapped hole at the distal end, placed accurately over the centre of the cup. The glass tube filled with blood was placed in a stout rubber band, a small brass cup was placed over the upper end of the tube the lower end of which was then pressed firmly into the rubber cushion in the bottom of the centrifuge cup, by means of a nut which passing through the tapped hole fitted into a narrow and comparatively deep hole in the brass cup.

This device (Fig. 1) was used in duplicate and after centrifuging the specimen of blood for 15 minutes very accurate measurements could be made of the proportion of cells to plasma in a length of 10 cm. of blood.

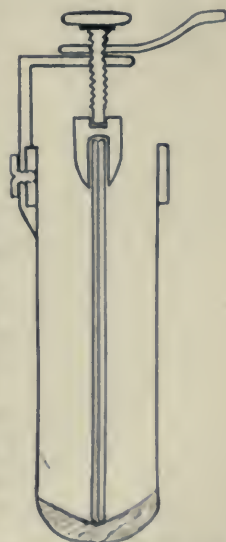


Fig. 1. A 50 cc. metal centrifuge tube adapted for obtaining haematocrit readings with a 10 cm. length of blood.

Table I. *The distribution of calcium in the cells and plasma of normal dogs.*

Dog	pH	Total Ca mg. %	Haematocrit reading mm.	Plasma Ca per 100 cc. blood	Cell Ca per 100 cc. blood	Cellular Ca % of total
I	7.5	9.60	3.7	8.36	1.24	12.19
II	7.5	8.1	3.6	7.20	0.90	11.1
III	7.5	8.00	3.4	7.26	0.74	9.3
IV	7.5	8.00	2.8	7.28	0.72	9.0
V	7.55	7.75	3.4	6.79	0.96	12.2
VI	7.55	8.10	3.1	7.24	0.86	11.6
VII	7.55	10.00	3.1	8.83	1.17	11.7
VIII	7.50	8.20	3.2	7.8	0.40	4.9
IX	7.4	7.50	3.2	7.20	0.30	4.0
X	7.5	7.85	3.5	6.95	0.90	10.2
XI	7.45	9.12	3.5	8.25	1.87	9.6
XII	7.45	9.60	3.0	8.40	1.20	12.5
XIII	7.45	10.80	2.7	9.56	1.24	11.5
XIV	7.5	11.00	3.4	9.90	1.10	10.0
XV	7.5	9.70	2.8	8.85	0.85	8.8
XVI	7.5	10.00	2.5	8.70	1.30	13.0

Table II. *The distribution of calcium in the cells and plasma of tetany dogs.*

Dog	pH	Total Ca mg. %	Haematocrit reading mm.	Plasma Ca per 100 cc. blood	Cell Ca per 100 cc. blood	Cellular Ca % of total
I	7.6	5.82	3.0	5.32	0.50	8.5
II	7.70	5.37	1.9	4.86	0.51	9.5
III	7.65	5.62	2.8	5.19	0.43	7.6
IV	7.75	5.75	3.2	5.16	0.51	10.26
V	7.6	6.5	3.5	6.04	0.46	7.1
VI	7.7	6.25	3.3	5.83	0.42	6.7
VII	7.6	6.50	3.6	6.1	0.40	6.2
X	7.65	4.93	3.5	4.45	0.48	9.7
XI	7.65	4.75	2.7	4.41	0.34	7.1

From Table I it will be seen that the average result of the total calcium of the blood of 14 dogs is 9.12 mg. %. In computing this average the figures for dogs Nos. VIII and IX are not included as these dogs were suffering severely from mange.

The average figure for the amount of calcium in plasma from 100 cc. of blood is 8.11 mg. while for the corpuscles from the same amount of blood it is 1.01 mg. that is to say in a hundred parts by volume of blood we find a distribution of calcium between the plasma and the cells in the proportion approximately of 8 to 1.

In a series of some twelve rabbits in which similar estimations were made it was found that the figures were slightly higher, being for whole blood 10.0 mg. %, for plasma from 100 cc. of blood, 8.6 mg. and for the cells 1.4 mg.

Estimations were performed upon the parathyroidectomised dogs usually on the third day after operation. If the animals survived for two or three days longer, a second determination was made. On several of these animals a partial parathyroidectomy was performed but no conclusive results were obtained immediately following such an operation and the results quoted are those in totally parathyroidectomised animals. The pH was estimated on the

day following operation on blood drawn from the left ventricle, the method used being that of Levy, Rowntree and Marriott [1915]. The alkaline reserve was determined by the colorimetric method of Marriott [1916]. The relation of the p_{H} to alkaline reserve will form the subject of a further communication in reference to the carbon dioxide combining power of the plasma of tetany dogs.

Average figures on seven completely parathyroidectomised dogs quoted in Table II show a whole blood calcium content amounting to 5.72 mg. %, with a plasma and cell content per 100 cc. of whole blood of 5.26 mg. and 0.46 mg. respectively. It is thus seen that in experimental tetany the ratio of plasma calcium to cell calcium is approximately 11.5 to 1.0.

Table III. *A comparison of the average figures for calcium distribution in normal and tetany dogs.*

Ca % in mg. whole blood	Plasma Ca mg. per 100 cc. of blood	Plasma Ca % of total Ca	Cell Ca mg. per 100 cc. blood	Cell Ca % of total Ca	Remarks
9.12	8.11	88.9	1.01	11.1	Normal dogs
5.72	5.26	91.9	0.46	8.1	Tetany dogs
37.4	35.2	—	54.4	—	Percentage fall in calcium

The alteration in the distribution may be more clearly seen from Table III, in which the relative percentage of calcium in the plasma and cells to the total blood calcium is shown and in which the comparative alterations are indicated as percentages of the normal contents. It is here shown that in the condition of tetany there is a considerable loss of cell calcium amounting to 54.4 %, as compared with a plasma loss of 35.2 %.

II.

OBSERVATIONS ON DIFFUSIBLE AND COLLOIDAL CALCIUM IN NORMAL AND PARATHYROIDECTOMISED DOGS.

In 1921 a method for the estimation of diffusible calcium was introduced by von Mysenbug and his colleagues [1921]. This method is one of compensation dialysis, in which the diffusible salt in the dialysee is balanced by the addition of an equal amount of the salt to the dialysing fluid.

The separation by filtration of the diffusible salts of blood serum can be effected by subjecting the filter to a pressure of 150 mm. of Hg. This method was employed by Cushny [1920], but to determine accurately the amounts of a salt in ionic and colloidal form, it is essential not to alter the balance between them by removal from the solvent of the diffusible salt. According to the law of mass action the removal of the ionic calcium would result in a further breakdown of calcium in combination in order to restore the normal balance. For this one reason therefore apart from others the compensation method of dialysis is the better and has been made use of in this work.

There are however difficulties associated with the method, the chief of which is the phenomenon of negative osmosis, a phenomenon in which water

may pass from the more concentrated to the more dilute side, that is against osmotic pressure. It may be mentioned here that in dialysing blood serum it is not always sufficient that the dialysate be a solution isotonic with blood serum.

Alterations in permeability of the membrane may be brought about by alterations in the membrane itself, by changes in the solutions within or without the membrane, and by changes in the electrical double layer on the surface of the membrane. If a solute of opposite electrical sign come within the sphere of electrical attraction of the membrane it will be adsorbed. If the adsorbed material be an amphoteric colloid the electrical charge on the membrane will be modified. It is this adsorption by membranes of amphoteric colloids which is responsible for the phenomenon of negative osmosis; water therefore will pass through the membrane at a rate depending upon the density, not the sign, of the electrical charge on the membrane.

The diffusion may be caused either by pure osmosis or by electrical osmosis, the latter being dependent upon the presence of electrolytes and also upon the fact that collodion membranes adsorb amphoteric colloids, thus producing electrical charges on the membrane.

Such salts as oxalates, citrates and phosphates attract water markedly but the effect of the anion may be neutralised by the cation, and this explains the weak action of calcium chloride where the electrical charge on the cation practically neutralises the two charges on the anions.

The difficulties associated with semi-permeable membranes are well known [see Burns, 1921 and Loeb, 1917].

It has been found that very thin sacs made of parlodion are too readily permeable to protein while sacs which are too thick are useless because of negative osmosis whereby the volume of the serum is increased. In such sacs the degree of permeability to electrolytes is also considerably reduced. In a very interesting paper on the degrees of permeability of collodion sacs Eggerth [1921] has shown that the speed of diffusion of electrolytes, "non-dialysable" colloids, like congo red, and proteins of serum and of blood cells depends upon the permeability of the membranes, and the relative permeability of the membrane depends upon the proportions in which alcohol and ether are present in the alcohol ether solvent used for dissolving the pyroxylin, or parlodion to give it the name under which it is manufactured. Sacs made by dissolving collodion in ether and alcohol in the proportion of 60 to 40 parts, were found to be impermeable to protein after three hours' dialysis. They still retained their maximal permeability to crystalloids, all of which were found to pass through in 15 minutes. To obtain sacs which will withstand dialysis of 24 hours at 15-20°, it is necessary to employ a "30 to 40 alcohol" membrane. With such a membrane the diffusion of electrolytes is complete in 10 to 15 minutes and it has been demonstrated that in the presence of protein the diffusion of calcium salts is complete well within 24 hours.

While a considerable drop in the total calcium of the blood and of its cellular elements has been noted in parathyroid tetany, the question as to

whether or not this was due to a loss in free or combined calcium has not been definitely settled. By dialysis we can determine the amounts of free and combined calcium in blood plasma or serum but no method has been perfected whereby a similar determination can be carried out with regard to the cellular elements of the blood.

That alterations may occur in the diffusible calcium in rickets and in tetany was suggested by von Mynsbug and his co-workers, but they in a series of four experiments found no distinct variations from the normal.

METHODS.

Preparation of parlodion sacs. The shreds of parlodion are dried in the air and dissolved in ether and 95 % alcohol in the required proportions. After solution of the parlodion shreds is complete, the bottle, firmly stoppered, is allowed to stand for two weeks.

Two small test-tubes 15 mm. internal diameter and 60 mm. long are filled with the mixture, centrifuged to bring all air bubbles to the surface, the hardened surface then peeled off and the contents emptied into the stock bottle, the tube being rotated meanwhile, in order to secure uniform thickness of the wall of the sac. The tube is clamped in the inverted position for 15 minutes, then placed in cold distilled water for half-an-hour. The upper rim is loosened by means of a knife and a thin glass rod with a bulbous tip gently pushed between the sac wall and the glass to the bottom of the test-tube, which is kept half filled with water. The sac and the glass rod are withdrawn together and the sac immersed in cold distilled water.

Compensation dialysis. In view of the fact that in parathyroid tetany variations in diffusible calcium may be great or small, it was necessary to make up a series of dialysing calcium solutions in order to balance as nearly as possible, the amount of free calcium found in the serum. For this purpose therefore ten dialysing solutions were made up giving a percentage of normal serum calcium varying from 40 to 80. These solutions were made from a stock solution of calcium chloride of about 1 % strength, the amount of calcium in the stock solution having been determined by the nephelometric method. The following is the series of solutions employed:

COMPENSATION DIALYSING SOLUTIONS.

Solution	Amounts of Ca in mg. in 2.5 cc.		Amounts of Ca in mg. %		% of normal Ca
	Estimated	Calculated	Estimated	Calculated	
A	0.206	0.193	8.25	7.71	39.28
B	0.213	0.210	8.54	8.40	40.65
C	0.243	0.241	9.75	9.65	46.42
D	0.257	0.262	10.30	10.50	49.10
E	0.290	0.289	11.62	11.58	55.33
F	0.312	0.315	12.50	12.60	59.52
G	0.343	0.338	13.75	13.50	65.47
H	0.367	0.363	14.68	14.70	69.90
I	0.390	0.355	15.62	14.19	74.38
J	0.406	0.420	16.25	16.70	77.38

To 2.5 cc. of the solution employed, 2.5 cc. of a double strength dialysing fluid were added the composition of which is as follows:

Dialysing fluid (Greenwald).

Salt	% amounts single strength g.	Molar strength	Amounts in 2000 cc. double strength
NaHCO_3	0.2520	0.003	10.08 g.
KH_2PO_4	0.0088	0.000065	12.9 cc. <i>M/5</i>
MgCl_2	0.01059	0.00011	0.2118 g.
KCl	0.02960	0.000397	1.184 g.
NaCl	0.6250	0.01069	25.0 g.

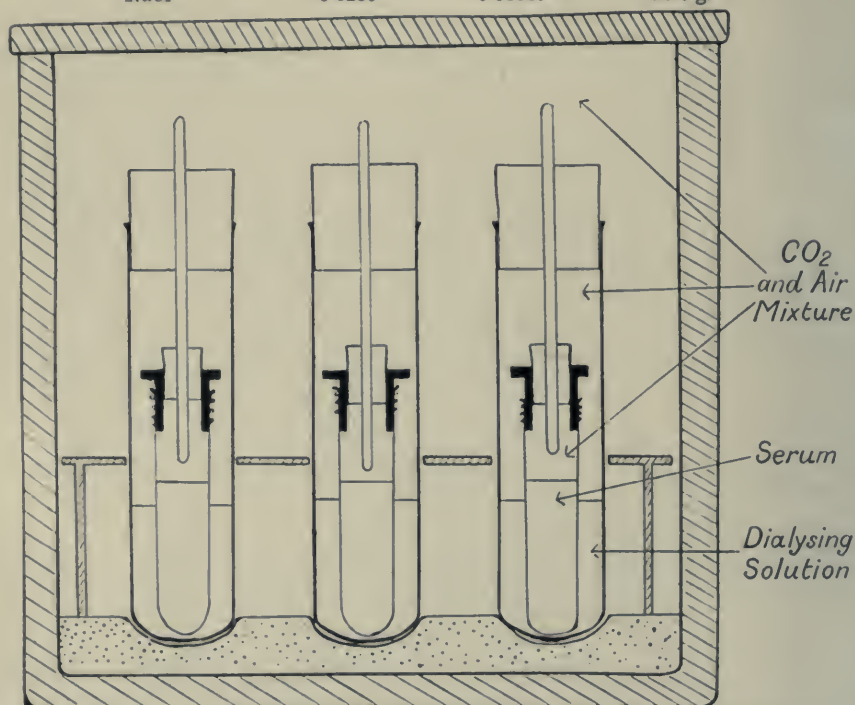


Fig. 2. Diagram of the method of setting up a series of six sacs for compensation dialysis of the blood serum.

Since the balance between diffusible and colloidal calcium is in some measure dependent upon the hydrogen ion concentration of the serum, it is essential to bring the CO_2 tension of the serum into equilibrium with an atmosphere of CO_2 of a tension approximating that of alveolar air. To do this a large spirometer was filled with CO_2 and air, these were thoroughly mixed, and the percentage of CO_2 estimated daily by the Henderson and Morriss [1917] method.

The gas was passed through a bottle containing moist glass beads then through two separatory funnels placed in series, the first containing about 20 cc. of serum the second about 10 cc. of the double strength dialysing fluid. This equilibration was carried out at room temperature which during the course of these experiments varied from 30 to 33°.

The dialysis. The sacs to be employed were thoroughly washed in distilled water, shaken free of water and allowed to drain, but not allowed to dry in the air. The test-tubes used for dialysis were $\frac{3}{4}$ " wide by 4" long and into these were placed 2.5 cc. of the double strength dialysing fluid, and CO₂ passed into the test-tubes for a few minutes.

The sacs supported on small hard rubber cylinders, were filled with 5 cc. of serum, CO₂ was passed into the sacs, a small cork firmly inserted into the top of each cylinder, and the whole supported in the test-tube as shown in Fig. 2. Dialysis was carried out for 24 hours at a temperature of 12 to 15°.

DISCUSSION OF RESULTS.

In the following series of Tables Nos. IV to VII, the results obtained in normal, partially and totally parathyroidectomised dogs are given.

Table IV. *Total calcium in normal, partially and completely parathyroidectomised dogs (mg. per 100 cc.).*

Normal	Partial	Complete
11.24	6.25	5.0
10.50	6.13	5.10
11.20	8.20	5.00
11.30		4.70
12.70	Average 7.04	5.20
12.60		4.60
9.00		5.40
Average 11.30		Average 4.95
100 %	→ drop 37.72 %	→ drop 56.20 %

Table IV shows the variations in percentage of the calcium content of serum under the above three conditions. From a normal average of 11.30 mg. %, the calcium fell in partially parathyroidectomised animals to an average of 7.04 mg. %, to be followed by a further fall upon complete parathyroidectomy to an average figure of 4.95 mg. %. It is thus seen that the loss of calcium from the serum amounts to 56.20 % of the whole.

With regard to diffusible calcium, the figures quoted in Table V, which comprises results obtained from a series of seven normal dogs, are comparable to those of Cushny and von Maysenbug, namely that in normal animals the diffusible calcium amounts to between 60 and 70 % of the whole calcium content of the blood taking 10.5 mg. as the percentage of calcium in normal blood serum.

In comparing the results recorded in Tables VI and VII, results from partially and completely parathyroidectomised dogs respectively, it is seen that there is no essential difference in the amounts of calcium recorded.

It is somewhat difficult to say to what extent a single parathyroid gland may or may not be functioning. The upper left parathyroid was left with a small piece of thyroid tissue. In all of these cases the gland retained was distinctly identified, but we must admit that this method of leaving a gland

RESULTS OF COMPENSATION DIALYSIS OF SERUM CALCIUM.

NORMAL DOGS.

Table V.

Dog I. p_{H} 7.5; Total Ca = 10.5 mg. %; Ca in 5 cc. serum = 0.525 mg.;					
CO ₂ tension = 49.64 mm. Hg.					
Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium	Compensation dialysing solution
Begin	End	Begin	End	%	
.525	.584	.347	.317	55.5	G
.525	.534	.343	.316	55.4	G
.525	.493	.312	.332	67.1	F
p_{H} 7.5; Total Ca = 11.24 mg. %; Ca in 5 cc. serum = 0.562 mg.;					
CO ₂ tension = 50.16 mm. Hg.					
.562	.503	.312	.337	64.4	F
.562	.516	.343	.413	61.4	G
.562	.462	.462	.383	70.6	H
Dog II. p_{H} 7.5; Total Ca = 10.5 mg. %; Ca in 5 cc. serum = 0.525 mg.;					
CO ₂ tension = 50.92 mm. Hg.					
.525	.530	.344	.344	65.5	G
.525	.515	.367	.354	64.9	H
Dog III. p_{H} 7.45; Total Ca = 11.7 mg. %; Ca in 5 cc. serum = 0.585 mg.;					
CO ₂ tension = 50.92 mm. Hg.					
.585	.505	.312	.342	63.5	C
.585	.558	.343	.362	65.1	G
.585	.550	.367	.382	67.9	H
.585	.557	.367	.377	66.1	H
Dog IV. p_{H} 7.45; Total Ca = 11.30 mg. %; Ca in 5 cc. serum = 0.565 mg.;					
CO ₂ tension = 44.14 mm. Hg.					
.565	.480	.312	.343	66.1	F
.565	.491	.343	.323	59.1	G
.565	.511	.367	.362	63.1	H
.565	.537	.367	.372	66.7	H
Dog V. p_{H} 7.4; Total Ca = 12.7 mg. %; Ca in 5 cc. serum = 0.635 mg.;					
CO ₂ tension = 42.56 mm. Hg.					
.635	.580	.212	.362	64.8	F
.635	.571	.343	.363	60.1	G
.635	.550	.367	.380	62.0	H
.635	.550	.367	.387	64.0	H
Dog VI. p_{H} 7.4; Total Ca = 12.6 mg. %; Ca in 5 cc. serum = 0.630 mg.;					
CO ₂ tension = 50.0 mm. Hg.					
.630	.590	.312	.332	55.9	F
.630	.589	.343	.352	56.8	G
.630	.621	.367	.372	59.3	H
.630	.599	.367	.367	57.8	H
Dog VII. p_{H} 7.4; Total Ca = 9.0 mg. %; Ca in 5 cc. serum = 0.450 mg.;					
CO ₂ tension = 49.4 mm. Hg.					
.450	.470	.312	.312	69.3	F
.450	.481	.367	.342	70.4	H

PARTIALLY PARATHYROIDECTOMISED DOGS.

Table VI.

Dog I. Total Ca = 6.75 mg. %; Ca in 5 cc. serum = 0.337 mg.; CO₂ tension = 50.0 mm. Hg.

Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium %	Compensation dialysing solution
Begin	End	Begin	End		
.337	.340	.210	.250	90.0	B
.337	.368	.257	.291	96.4	D
.337	.363	.312	.296	83.1	F
.337	.368	.367	.352	100.0	H

Dog II. Total Ca = 6.13 mg. %; Ca in 5 cc. serum = 0.306 mg.; CO₂ tension = 47.83 mm. Hg.

.306	.335	.257	.302	103.5	D
.306	.302	.257	.272	93.2	D
.306	.352	.312	.293	90.0	F
.306	.291	.406	.372	109.0	J

Dog III. Total Ca = 8.2 mg. %; Ca in 5 cc. serum = 0.410 mg.; CO₂ tension = 48.64 mm. Hg.

.410	.410	.257	.281	84.5	D
.410	.391	.367	.392	101.6	H
.410	.391	.406	.412	101.9	J

COMPLETELY PARATHYROIDECTOMISED DOGS.

Table VII.

Dog I. July 21, operation; July 22, hyperpnoea of Cheyne-Stokes type with salivation, fine tremor of muscles, tetany not severe; 70 cc. of blood drawn from femoral artery.

Total Ca = 5.0 mg. %; Ca in 5 cc. serum = 0.250 mg.; CO₂ tension = 49.4 mm. Hg.

.250	.314	.312	.280	99.2	F
.250	.246	.257	.221	90.0	D
.250	.310	.367	.312	102.8	H
.250	.291	.406	.322	95.2	J

Dog II. July 20, complete operation at noon; July 21, 2 p.m., tremors of neck and shoulder muscles, increased respiration with slight salivation; 8 p.m., marked tremors of muscles of the neck and of the hind limbs. Respirations very rapid and of Cheyne-Stokes type; 9 p.m., 75 cc. of blood drawn aseptically from the femoral artery under ether anaesthesia; 10 p.m., animal much improved, able to stand and able to drink on account of reduced respiratory rate; animal mentally alert.

Total Ca = 6.09 mg. %; Ca in 5 cc. serum = 0.305 mg.; CO₂ tension = 41.8 mm. Hg.

.305	.279	.257	.272	69.0	D
.305	.273	.312	.291	90.0	F
.305	.273	.367	.322	90.3	H

July 22, dog in marked tremor, capable of standing but legs stiff; respiration very irregular and rapid with a rate of over 200 per minute; 70 cc. of blood taken aseptically under ether; respiration rate normal under ether. Definite improvement for 36 hours after withdrawal of blood, the dog becoming quite lively and responsive. Tremors commenced within 36 hours, being felt over the shoulder-girdle; respiration rate again rapidly rising from 60 per minute to 180 per minute.

Total Ca = 5.1 mg. %; Ca in 5 cc. serum = 0.255 mg.; CO₂ tension = 44.84 mm. Hg.

.255	.287	.312	.261	82.0	F
.255	.299	.406	.322	93.4	J

Dog III. Total parathyroidectomy performed July 21; dog died in the night about 20 hours after operation, having developed hyperpnoea and slight tremor during the evening of the 21st; unable to obtain blood from veins because of clotting.

Dog IV. July 20, total parathyroidectomy; July 26, this dog has shown no signs of tetany.

Total Ca = 8.3 mg. %; Ca in 5 cc. serum = 0.415 mg.; CO₂ tension = 51.68 mm. Hg.

.415	.358	.367	.332	72.4	H
.415	.425	.257	.262	64.4	D

August 5, no signs of tetany have developed. Total calcium = 7.5 mg. %.

Dog V. July 22, total parathyroidectomy performed; dog in excellent condition. July 23, slight hyperpnoea, muscular twitching, and salivation developing. July 24, all the muscles of the abdomen, shoulders, head and neck and legs in constant and rapid twitching; dog is very thirsty but unable to drink because of the very rapid respiration rate amounting to 240 per minute; the dog is able to stand, is constantly on the move and constantly attempting to drink. July 24, 10 a.m., because of the severity of the condition 70 cc. of blood were drawn from the femoral artery; at 2 p.m. the condition had greatly improved; all tremors had gone and respirations were almost normal. July 26, noon, tetany attack developing with rapid respirations, salivation, muscle tremors and spasticity of the limbs. July 27, 9 a.m., severe tetany has developed with strong spasmodic contractions of muscles of the head and jaw, the latter causing sharp snapping of the teeth together, causing the tongue to be bitten. Respiration rate 250 per minute; salivation and thirst marked; animal lying soaked in perspiration. 11 a.m., because of the distressing condition animal bled to death.

July 24, Total Ca = 5.0 mg. %; Ca in 5 cc. serum = 0.250 mg.; CO₂ tension = 47.12 mm. Hg.

Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium %	Compensation dialysing solution
Begin	End	Begin	End		
.250	.313	.257	.254	100.4	D
.250	.313	.312	.282	100.8	F

July 26, Total Ca = 4.7 mg. %; Ca in 5 cc. serum = 0.235 mg.; CO₂ tension = 46.36 mm. Hg.

.235	.256	.257	.246	100.0	B
.235	.268	.312	.271	97.8	C

July 27, Total Ca = 4.7 mg. %; Ca in 5 cc. serum = 0.235 mg.; CO₂ tension = 42.56 mm. Hg.

.235	.305	.315	.227	96.6	F
.235	.332	.367	.300	99.1	H

Dog VI. July 22, total parathyroidectomy performed. July 30, usual signs of tetany; 70 cc. of blood taken from femoral artery for estimations and animal bled to death.

Total Ca = 4.6 mg. %; Ca in 5 cc. serum = 0.230 mg.; CO₂ tension = 50.16 mm. Hg.

.230	—	.312	.263	93.1	F
.230	.322	.367	.284	87.4	H

Dog VII. July 29, total parathyroidectomy performed; very definite signs of tetany developed within 48 hours.

Total Ca = 5.2 mg. %; Ca in 5 cc. serum = 0.260 mg.; CO₂ tension = 50.16 mm. Hg.

.260	.269	.257	.251	94.3	D
.260	.307	.367	.312	98.8	H

Dog VIII. Aug. 2, noon, total parathyroidectomy performed. Aug. 3, 9 a.m., marked tremor in muscles of the head and neck, shoulders, abdomen; respiration rate 220 per minute; 70 cc. of blood taken from femoral artery for estimations. Aug. 4, condition relieved temporarily by withdrawal of 70 cc. of blood. Aug. 5, 2 p.m., tremors of muscles very marked; perspiration and salivation marked; respiration rate about 200 per minute. 7 p.m., tremors of muscles as at 2 p.m.; haemorrhage from the tongue due to injury from spasmodic contraction of jaw muscles; perspiration profuse; spasticity of limbs marked; animal unable to stand; condition very severe.

Aug. 3, Total Ca = 4.6 mg. %; Ca in 5 cc. serum = 0.230 mg.; CO₂ tension = 50.16 mm. Hg.

.230	.280	.312	.241	73.5	F
.230	.290	.312	.240	73.0	F

Aug. 4, Total Ca = 6.0 mg. %; Ca in 5 cc. serum = 0.304 mg.; CO₂ tension = 51.68 mm. Hg.

.304	.310	.312	.302	97.6	F
.304	.309	.367	.283	98.5	H

Aug. 5, Total Ca = 5.4 mg. %; Ca in 5 cc. serum = 0.270 mg.; CO₂ tension = 49.56 mm. Hg.

.270	.179	—	.120	88.8	No compensa-
.270	.138	—	.126	93.3	tion fluid used

NOTE. The percentage of calcium which is diffusible calcium is calculated as follows:

$$\text{Diffusible calcium} = \frac{\text{Ca in dialysate after dialysis} \times 2 - \text{Ca added}}{\text{Original calcium in serum}}.$$

in situ while all other similar tissue is removed is not par excellence the method that should be followed in the investigation of the internal secretion of any gland. Just as in partial depancreatization so in partial parathyroidectomy, the gland or piece of gland should be transplanted and the activity of the graft definitely proved before it is totally removed. In the former method one can not with assurance state whether or not there has been any alteration in the normal blood and lymph supply due to operative interference. In all of these operative animals subcutaneous suture was employed, no dressings were applied and without exception excellent healing of the neck wounds was obtained.

It seemed that as far as the purpose of the investigation was concerned no practical end would be served by the method of partial parathyroidectomy: it was therefore resolved to perform the complete operation on all other animals in one stage.

From the average percentage of diffusible calcium recorded for these tetany animals namely 93.70, a percentage which is computed from the total amount of calcium in each sample of serum, it is evident that the loss of colloidal calcium is indeed a marked one.

It is true that the compensation in dialysing was not complete, but in view of the high percentages recorded and also in view of the results obtained on the final serum from dog VIII, in which no compensation was attempted, one is forced to the conclusion that in severe tetany the major part, if not all, of the colloidal calcium is freed from its protein combination. It would also seem from certain of the results that it would be possible to determine the rate of the breakdown of this calcium proteinate linkage. It appears however that the rapid loss of diffusible calcium from the blood demands a correspondingly rapid liberation of calcium from its colloidal state.

The great demand of the organism for calcium one can understand upon witnessing the severity of the condition. It seems feasible therefore to postulate that there is an attempt on the part of the organism to supply the bivalent salts necessary for the maintenance of a normal condition of irritability of the nervous system and the method adopted is that of a rapid liberation in ionic form of all the colloidal calcium in the blood. But unfortunately in virtue of its free state in the circulation the calcium is very readily lost by way of the kidneys, which organs being devoid of judgment and exercising no discrimination are not to be expected to respond to the abnormal condition by setting up any mechanism of curative adaption. Under these conditions it is evident that once the store of colloidal calcium in the blood is utilised, there must be a call upon the calcium of the tissues. That there is a muscle protein breakdown in tetany has been amply proved by several workers, more notably Paton, Findlay and Burns. It is possible therefore that there is a continual breakdown of muscle protein, which is responsible for the production of a toxic substance, and it is this steady decomposition of protein which is the mechanism whereby an attempt is made to meet the call of the nervous and other tissues for calcium.

In view of the urgent needs of the organism and probably as a result of the protein decomposition, the calcium is thrown out in that form in which it is most easily utilised.

In considering the reason for the presence of diffusible calcium in the blood in tetany the somewhat contradictory facts that both hydrochloric acid and calcium salts are effectual in the treatment of tetany open up a suggestion that the increase in the hydrogen ion concentration of the blood during acute tetany may be a means whereby more calcium can be liberated from protein combination. The lessened alkalinity of the blood would favour the dissolving of the calcium, and would thereby make immediately available for the body calcium as soon as it was broken out from its protein combination.

It must not be forgotten that the alteration in calcium metabolism is a sign of tetany, and that the causative factor is probably a body associated with the creatine of muscle protein.

Watanabe [1918, 1] has shown that the administration of guanidine induces severe acidosis, with a retention of phosphates and a decrease of calcium in the blood, and he expresses the possibility that the fundamental cause of tetany is the increased formation of guanidine nitrogen brought about by the disturbance of the function of the parathyroid glands. But he also points out elsewhere [1918, 2] that the tetany due to guanidine injection is not abolished by calcium administration. This last observation is contradictory to that of Fühner [1915] in which he states that the administration of calcium removes the effects of guanidine, an observation which tends still further to correlate these two conditions namely parathyroid and guanidine tetany. A factor in favour of the toxic theory which has been fully proved during this investigation is that the withdrawal of blood amounting to about 100 cc. and the injection of about 200–300 cc. of calcium-free saline, immediately effects an amelioration of the symptoms of tetany.

THE SIGNS OF PARATHYROID TETANY.

In all of these dogs with the single exception of No. IV definite symptoms of tetany developed. It is evident from the figures for dog IV that there was no derangement of the free and combined calcium balance, but it will be noted that there is a slight though definite loss in total calcium.

Dogs II, V, VI, VII and VIII developed signs of tetany within 36 hours and it will be seen from the notes in the tables that the attacks were with only two exceptions of a severe type. None of these animals suffering from severe tetany survived for more than five or six days. The signs noted as definitely indicating parathyroid tetany were the increased respiratory rate of from 200 to 250 per minute, with noisy expiration, often of a Cheyne-Stokes type, the profuse salivation, and nasal secretion, all of which developed within 48 hours. The characteristic muscle tremors were invariably in evidence within 24 hours as fine muscle twitchings which could be felt most clearly over the shoulder girdle. These tremors became more and more marked until

in some cases by the end of the second day after operation they had become coarse spasmodic twitchings. These were markedly in evidence in dogs V, VII and VIII, in which cases the spasmodic contractions of the temporal and masseter muscles were undoubtedly distressing, the latter causing injury to the tongue by the sudden snapping together of the teeth.

The spasticity of the limbs, on account of which the animal is forced to lie on its side, the inability to stand, the cardiac irregularities, vomiting, and at times profuse perspiration and loss of appetite were all in evidence two and at the latest three days after operation.

The mild or more chronic types of tetany described by others in which the animals lived for two or three weeks after operation have not been seen throughout this investigation; all were definite, and most were severe cases of tetany terminating fatally within one week. The severity of these attacks was always greatly ameliorated by the withdrawal of the 70 cc. of blood for the necessary examinations.

This severe condition occasioned by complete removal of the thyro-parathyroid apparatus may well account for the results recorded.

THE HYDROGEN ION CONCENTRATION AND CO_2 -COMBINING POWER OF THE BLOOD IN TETANY.

The p_{H} and the CO_2 -combining power of the blood were determined in these dogs. The normal p_{H} was found to vary between 7.45 to 7.50 while within 24 hours after parathyroidectomy it was usually somewhat higher varying from 7.55 to 7.75 but with the rapid development of the tetany it invariably fell to about or below the normal and after the third day it varied from 7.2 to 7.35.

In accordance with the findings of others the signs of initial alkalosis were undoubtedly present. Following this slight increase in the p_{H} , there was an indication that with the falling p_{H} the alkaline reserve was encroached upon by non-volatile acids, and as a condition of acidosis is produced the alkaline reserve becomes more and more depleted, until subsequently the compensatory loss of carbon dioxide by way of the alveolar air, whereby the p_{H} is maintained normal, is of no avail and the p_{H} falls, and continues to fall until the death of the animal.

That such a process may take place is suggested by the figures for the CO_2 -combining power of the blood which have been obtained. As far as the CO_2 -combining power of the plasma is concerned the high figures of McCann [1918] have not been confirmed.

The graph (Fig. 3) shows the average findings for CO_2 -combining power and the p_{H} of the blood arrived at in this laboratory. This aspect of the problem still requires further investigation, and it is hoped that the results of further research on this part at present being undertaken here will shortly be published.

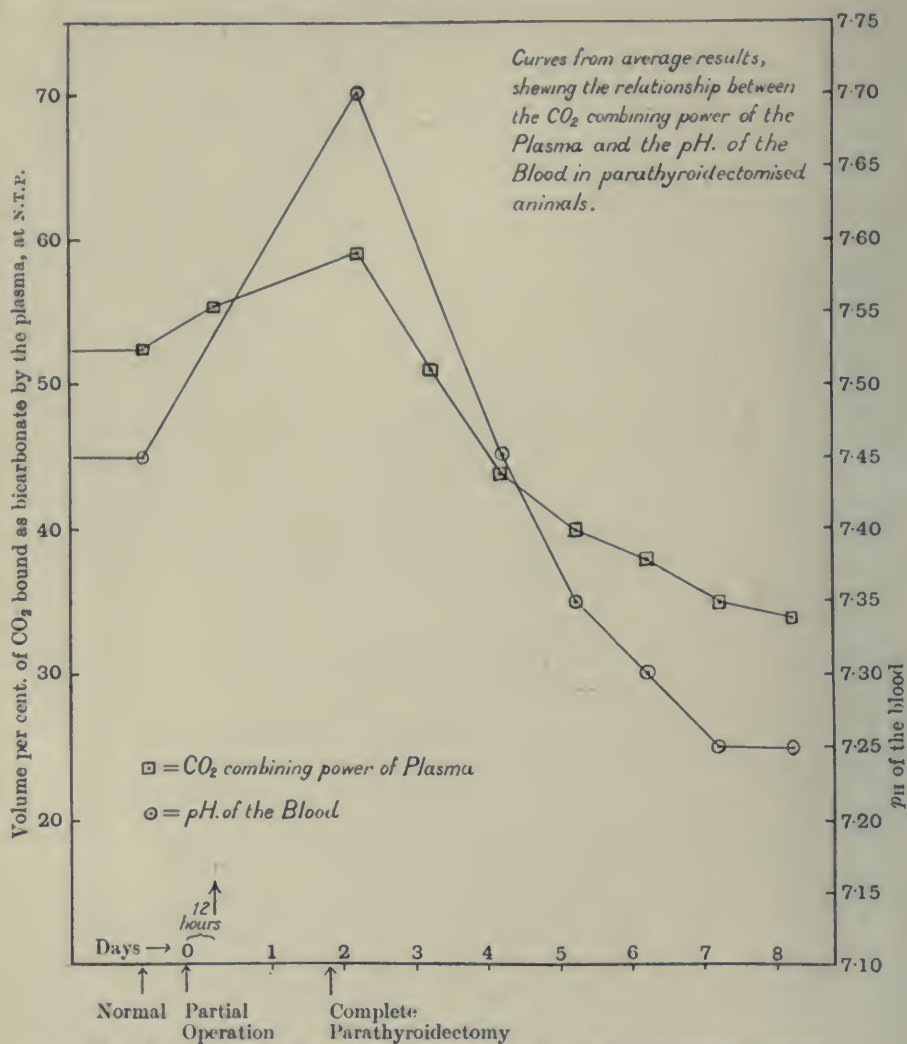


Fig. 3. Chart showing average results for the CO_2 -combining power of plasma and the pH of the blood.

SUMMARY.

(1) The calcium content of 100 cc. of normal blood amounts to the following average figures (in mg.), total 9.12, plasma 8.11, cells 1.01; in tetany these drop to the average figures of whole blood, 5.7, plasma, 5.26 and cells, 0.46. These figures show a loss of calcium amounting to 37.2 % for whole blood, to 54.4 % for the cells and 35.2 % for the plasma.

(2) Diffusible calcium in normal serum averages from 60 to 70 %, while in severe parathyroid tetany it amounts to 94 % of the total calcium.

(3) While there is an immediate state of alkalosis following parathyroidectomy, this condition is not necessarily marked and with the development

of the signs of severe tetany it passes rapidly into a condition of acidosis as shown by a steadily falling p_{H} of the blood.

(4) The immediate relief of the condition consequent upon the withdrawal of 70–100 cc. of blood is indicative of a toxic causative factor.

(5) The calcium deficiency and the great loss of colloidal calcium is merely indicative of a rapid protein disintegration.

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III. THE BACTERICIDAL ACTION OF TELLURIUM DERIVATIVES OF CERTAIN ALIPHATIC β -DIKETONES.

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(Received December 22nd, 1922.)

IN a previous paper [Morgan, Drew and Cooper, 1922] attention was drawn to the powerful inhibitory effect of the tellurium derivatives of aliphatic β -diketones upon the growth of *B. coli communis*. Attempts made to estimate the phenol coefficients of these substances in the ordinary way showed that on sub-culturing the broth tubes into fresh broth growths occurred, indicating that the bacilli had not been necessarily destroyed by the short exposures, e.g. 30 minutes, to solutions of the tellurium compounds. The phenol coefficients could not therefore be estimated accurately, but indirect evidence was obtained of a strong inhibitory action, and it was calculated that in the case of tellurium propionylacetone the inhibitory effect would be appreciable even in a concentration of 1 in 5,000,000.

These tellurium compounds would, therefore, be classified in the usual way as growth-inhibitors, rather than as disinfectants, but it is probably more correct to regard them as substances which possess the remarkable property of slowly exerting a marked germicidal action in extremely low concentration, and in this respect they differ fundamentally from phenols and other substances which are only active in much higher concentration.

Further experimental work has since been carried out with the following objects in view:

1. To ascertain the influence of chemical constitution on the germicidal power of these tellurium derivatives, with the object of obtaining substances of definite chemical structure fulfilling the requirements of high germicidal power, and minimum toxicity to higher animals.
2. To investigate the specific action of tellurium compounds on different micro-organisms.

For this work the usual method of determining phenol coefficients has been superseded by the following process, in which a comparative basis has been introduced as an essential condition of the inhibitory method.

Experimental method.

To constant volumes (8 cc.) of Lemco-peptone broth (acidity = + 7E) varying volumes of sterile distilled water were added, so that on the subsequent addition of requisite amounts of the tellurium β -diketone solutions, the total volume would in each case be 10 cc. Before the addition of the germicidal substance, however, one drop¹ of a 24 hours' culture of the test-organism was added to each tube. After thoroughly mixing, the disinfecting solutions were added, and after repeated mixing the tubes were incubated at 37° for 48 hours. In this way a definite number of organisms were subjected to the action of the various concentrations of germicide for a definite period in the presence of a sufficient food supply. For each experiment a series of phenol control-tubes was set up in a similar way and at the end of 48 hours the phenol coefficients could thus be calculated.

This method of experiment conforms more closely with actual conditions and is thus of more practical value than the ordinary phenol coefficient test in investigating the action of germicides of possible use for internal application, inasmuch as the disinfectant is allowed to act slowly over a considerable period at body temperature and in the presence of organic matter.

It should be pointed out that if the culture tubes were subcultured into fresh broth, no growths occurred on subsequent incubation. This result demonstrates that in these experiments the tellurium compounds were really exerting a bactericidal action, and were not only growth-inhibitors.

The phenol coefficients have previously been expressed in terms of concentrations of *grams per cc.* We consider it, however, more accurate to calculate the results in terms of molecular weights, and our results are therefore set forth as equimolecular phenol coefficients, *i.e.*

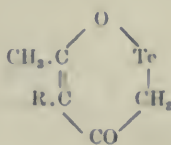
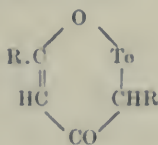
$$\text{phenol coefficients} \times \frac{\text{molecular weight of substance tested}}{\text{molecular weight of phenol}}.$$

In certain cases considerably different results are obtained.

The β -diketones employed in producing the tellurium compounds are acetylacetone and its homologues with the substituted alkyl group attached either to a terminal carbon atom or to the central one (position 3) as shown by the following formulae:



The general structures of the corresponding types of tellurium derivatives are to be represented as follows:



¹ We have employed smaller and also larger numbers of organisms in different experiments, but the results are not appreciably affected by variation in numbers.

For convenience of terminology these substances are referred to in the following table as derivatives of methane.

The organism employed in the experiments was *B. coli communis*, and it was found that while the resistance to phenol was remarkably constant, that of the same organism to the tellurium compounds varied considerably. For this reason the results in column II in the following table are expressed as ranges of concentration through which the substances were found to be bactericidal, while in column III a characteristic mean concentration is set forth. For convenience the concentrations are expressed in grams per cc. but in column IV the phenol coefficients are "equimolecular" coefficients and represent accurately the relative germicidal powers of the substances examined.

Table I.

	Substance	Range of minimum concentration bactericidal to <i>B. coli</i>	Mean bactericidal concentrations	"Equimolecular" phenol coefficients
1.	Tellurium diacetylmethane	1 in 270,000- 750,000 (4) ¹	1 in 500,000	2,800
2.	" 3-methyldiacetylmethane	1 in 550,000- 1,000,000 (4)	1 in 900,000	4,000
3.	" 3-ethyldiacetylmethane	1 in 1,250,000- 3,500,000 (6)	1 in 2,500,000	13,000
4.	" acetylpropionylmethane	1 in 1,000,000- 5,500,000 (5)	1 in 3,000,000	14,000
5.	" dipropionylmethane	1 in 7,000,000-10,000,000 (6)	1 in 9,000,000	45,000
6.	" acetylbutyrylmethane	1 in 1,000,000- 5,500,000 (10)	1 in 3,000,000	15,000
7.	" propionylbutyrylmethane	1 in 1,500,000- 4,500,000 (4)	1 in 2,800,000	14,000
8.	Phenol	1 in 510- 590	1 in 550	—

¹ The figures in brackets indicate the number of tests.

A consideration of the tabulated results shows that these tellurium compounds possess an extraordinarily high bactericidal power. The equimolecular phenol coefficients for the different members of the series range from 2,800 to 45,000 even in the presence of organic matter, the most active member being tellurium dipropionylmethane, which is bactericidal in a dilution of 1 in 9,000,000.

The bactericidal action increases considerably as the homologous series is ascended, until with a third substitution of the methyl group (as in tellurium propionylbutyrylmethane) a limit is reached and there is even a diminution in germicidal activity.

The efficacy of the compounds is furthermore influenced by the chemical structure of the β -diketone, position isomerism playing a very important part in determining bactericidal power. Thus, tellurium acetylpropionylmethane and tellurium 3-methyldiacetylmethane are isomeric, the methyl group in the former being attached to a terminal carbon atom, in the latter to the central atom, in position 3. Tellurium dipropionylmethane and tellurium 3-ethyl-diacetylmethane form a similar pair of isomerides. In each case it is seen that a terminal substitution of methyl group enhances bactericidal power to a very much greater extent than when substitution is effected centrally.

Again, tellurium dipropionylmethane, tellurium acetylbutyrylmethane and

tellurium 3-ethyldiacetylmethane are all three isomeric, the chemical constitution of the three β -diketones from which they are derived being respectively

- (1) $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_3$,
- (2) $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$,
- (3) $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}(\text{C}_2\text{H}_5) \cdot \text{CO} \cdot \text{CH}_3$.

The results set forth in Table I show that the tellurium compound of the symmetrical β -diketone (1) is much more efficacious than that of the dissymmetrical isomeride (2), which in turn is somewhat more active than the third isomeride, where substitution has been effected entirely in the central position.

Results of much the same order have been obtained with a different strain of *B. coli*.

A few experiments with the following organisms have also been carried out: (1) *B. paratyphosus A* and *B*; *B. typhosus*; (2) *Streptococcus haemolyticus*, *Staphylococcus pyrogenes aureus*, and the results so far indicate that the foregoing tellurium compounds exert a selective action on coliform organisms, the *cocci* on the other hand being much more resistant. In this respect the substances resemble benzoquinone [Cooper, 1912; Morgan and Cooper, 1921] and telluric acid [Joachimoglu, 1922] which has been found by the latter to be selectively inhibitory to coliform organisms in a concentration of 1 in 40,000.

The authors are extending the investigations to other pathogenic organisms including the acid-fast group, and, with the object of ascertaining whether these tellurium compounds can be employed for the purpose of inner disinfection, experiments are shortly being carried out to determine their toxicity to higher animals.

The authors desire to express their thanks to the Advisory Council of the Department of Scientific and Industrial Research for grants which have helped to defray the expenses of this investigation.

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IV. CALCIUM CONTENT OF THE BLOOD DURING PREGNANCY.

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THE importance of calcium in growth is well established, and in the human foetus it has been shown that calcium retention varies very greatly at the different stages of development.

Paul Bar [1907] by estimating the amount of calcium found in foetuses of different ages concluded that the absorption varied considerably with the stage of growth and was of the order shown in the following table (calcium expressed as calcium oxide):

0.0048 g.	each day up to 120th day.
0.083	„ „ from 120th-150th day.
0.087	„ „ „ 150th-180th „
0.084	„ „ „ 180th-210th „
0.638	„ „ „ 210th-270th „

From these figures it would appear that at certain periods there is an abrupt alteration in the calcium retention which must mean a sudden change in the demands upon the maternal stores of that element.

The question then arises does the blood of the mother show at these stages an alteration in its calcium content (an increase if the blood carries more calcium to meet the heavier demand made upon it, or a decrease if the demand is not counterbalanced by a greater mobility of the calcium), or is the calcium metabolism so perfectly balanced that the blood maintains a constant value in spite of the varying demands made upon it? In an attempt to answer this question, a number of analyses of the blood of women at different stages of pregnancy have been made, paying particular attention to those periods at which the abrupt changes in the calcium metabolism of the foetus seem to occur. The samples were taken 3rd-4th month, 5th month, 6th-7th month, and 8th-9th month.

METHOD.

The analysis was always made on serum, and not on the whole blood. The calcium is present in very different amounts in the corpuscles and plasma, and unless therefore the proportion of corpuscles to plasma is ascertained, the true value of the calcium content cannot be arrived at. Some of Lyman's varying results may be due to his always making use of whole blood. The method finally adopted was based on that of McCrudden [1912] modified for small quantities.

The working out of this method was started in 1919, and it was found that a similar method was being adopted by other workers for estimating small quantities of calcium. 5 cc. of serum were found to be sufficient and concordant analyses could be made when this amount was available.

Precipitation of the protein.

The protein of the serum is precipitated with 6.5% trichloroacetic acid, the volume taken being five times that of the serum, a rather larger ratio than that used by Lyman [1917, 1, 2]. The serum is added very slowly drop by drop from a pipette to the trichloroacetic solution in a small flask, rotating the liquid continuously. The flask is then stoppered and the contents thoroughly mixed by shaking. After standing for about half-an-hour, the mixture is filtered through a very small calcium-free filter paper, and an aliquot part of the filtrate is taken for the estimation of the calcium present.

Precipitation and estimation of the calcium.

The aliquot part of the filtrate is made just alkaline with concentrated ammonia, added drop by drop, using methyl red as indicator, then just acid with $N/2$ hydrochloric acid, added drop by drop from a burette or 1 cc. pipette. To this is added 1 cc. of $N/2$ hydrochloric acid, 1 cc. of oxalic acid solution 4%, and 1 cc. of a 20% sodium acetate solution, stirring or shaking carefully while adding the latter. The solution is allowed to stand for a day, and the precipitate of calcium oxalate is separated by means of a high speed centrifuge and washed twice with water, centrifuging between each washing. The calcium oxalate so obtained is dissolved in 4 cc. of N sulphuric acid made just pink with $N/100$ potassium permanganate, heated to about 70° on a water-bath, and then titrated with $N/100$ potassium permanganate solution, made according to the direction of Halverson and Bergeim [1917]. In this estimation of course all materials used must be tested for calcium, and only calcium-free materials employed. The water is always twice distilled and every care must be taken throughout the estimation to protect the solutions from contamination with dust or other reducing matter. Kramer and Howland [1920] described a method for estimating calcium in 1 cc. of serum, and on testing this method satisfactory results were obtained. This method was therefore occasionally adopted when only 2 cc. of serum were available, duplicate estimations always being made.

RESULTS.

The first series of analyses were made during 1920 to 1921, and in these there was no attempt to follow the same case throughout the different stages. The figures for this series, given below in Tables I and II are therefore the mean of experiments made on different women at the same period of pregnancy. The specimens of blood were obtained from maternity patients attending at the Royal Free Hospital.

The amount of calcium in the following tables is always given in milligrams of calcium per 10 cc. of serum.

Table I. (1st Series 1920-21.)

Stage month	Number of specimens analysed	Average calcium found	Range of calcium found	
			Highest	Lowest
3-4	12	0.968	1.17	0.763
5-5½	10	1.00	1.29	0.72
6-7	29	1.02	1.2	0.72
8-9	15	0.966	1.25	0.74

The above table shows little variation between the highest and lowest amounts of calcium in 10 cc. of serum at the different stages. Usually the range is from 1.05 to 0.93. These results seem to bear out De Wesselow's conclusion [1922], that during the last months of pregnancy there is a tendency to a fall in the calcium content of the serum. The results above are taken from all the cases examined during the year 1920-21 without separating the normal cases of pregnancy from those who in previous pregnancies had had miscarriages or still-born infants. In Table II this separation has been made.

Table II.

Stage month	Number of specimens analysed	Average calcium found normal	Numbers of specimens analysed	Average calcium found in cases previously abnormal
3-4	10	0.97	2	0.96
5	4	1.13	6	0.916
6-7	25	1.024	4	0.989
8-9	11	0.957	4	0.992

The numbers in Table II show that in the normal cases there is a decided drop in the calcium content during the last month of pregnancy, while in the other cases the difference in the calcium content at the different stages is not so marked, the calcium being slightly higher in the last month than at the beginning. The number of these latter cases is small, and a larger and more complete set is being investigated.

From the wide range of values for any given month in different cases it was clearly seen that for a definite answer to the problem the analyses must be made on the *same* individual at the different periods. A second series of analyses on these lines was carried out during the year 1921-22. In this way compensation was made for any individual idiosyncracies causing a variation

in results. In the second series in as many cases as possible analyses of the blood calcium have also been made within 14 days after confinement to determine the effect of lactation on the calcium content. Analyses are also now being made of the blood calcium of cases 2-3 months after confinement to investigate the effect of continued lactation. These results are given in Table III.

Table III. (2nd Series 1921-22.)

Stage Month	Number of specimens analysed	Average calcium found	Range of calcium found	
			Highest	Lowest
3-4	22	1.11	1.28	0.898
6-7	24	0.996	1.17	0.848
8-9	24	0.94	1.03	0.82
Post-confinement within 14 days	18	0.994	1.15	0.814

These results are in accord with those of last year and show a decrease in the calcium content of the serum during the last month of pregnancy, and a tendency to rise after confinement and during the early stages of lactation.

As stated earlier in this paper, during this year an endeavour was made to obtain the calcium content of the serum of the *same* woman at the above different stages of pregnancy, and this has been successfully carried out in a number of cases. In some, however, the series are not complete owing to the women failing to come to the hospital at one or other of the periods under investigation. The more complete figures are given in Table IV.

Table IV.

Case	Month 3-4	Month 6-7	Month 8-9	Post-confinement
4	1.23	1.1	—	1.07 (10 days)
7	1.09	0.916	0.775	1.07 (10 days)
8	0.898	0.93	0.92	—
9	—	1.17	1.00	—
12	1.17	0.88	—	1.05 (5 days)
13	0.913	0.898	0.92	1.01 (6 days)
14	1.2	0.948	—	1.04 (10 days)
16	—	0.944	0.96	0.814 (13 days)
17	—	1.15	0.866	—
18	—	0.97	0.82	0.999
20	1.17	0.943	0.91	0.999 (7 days)
22	—	1.03	0.93	1.06 (13 days)
23	1.17	1.11	0.983	—
29	—	0.85	1.03	—
30	—	0.943	0.897	0.889
36	—	0.91	—	0.999 (13 days)
38	—	0.95	—	1.02 (7 days)
41	0.93	1.07	0.88	—
42	—	—	0.984	1.15 (14 days)
44	—	0.856	0.92	—
45	—	—	0.79	0.933 (10 days)

From a study of these numbers it is seen in almost all the cases there is tendency to a decrease of calcium content of the blood toward the last months of pregnancy and a general tendency to rise directly after confinement. In

cases 13, 16, 29, 44 the results do not seem to be in accord with this general tendency, but in 13 and 16, the calcium content of the serum throughout pregnancy varies very little. These variations may be due to some other concurrent condition of the system at the time of observation. In one case of nephritis recently investigated the calcium content of the serum was found as low as 0.32 mg. per 10 cc.

Other workers in this field do not seem to have observed the above regular rise and fall in the calcium content of the blood at the different stages of pregnancy. Jansen [1918, 1, 2] thinks that the calcium content is little altered by pregnancy. He worked however on the whole blood and the disadvantages of this have been already indicated. Lamers [1912] thinks that the calcium content in blood in pregnant women is higher than that of non-pregnant women. Blair Bell [1908] showed in experiments on hens that the calcium content of the blood always rose previous to the laying of an egg and then immediately fell, there being a continuous rise and fall in the case of a hen that laid an egg on alternate days. According to this the calcium content of the blood of a woman ought to rise immediately before the birth of the infant and drop immediately after. The estimates given in the previous tables have not been made later than during the 8th and 9th month—never the day before the confinement. Also the earliest time after confinement that specimens of blood have been obtainable is five days. It would be interesting to examine the blood the day before and the day after confinement in the light of Bell's results.

The fact that the foetus is constantly taking in larger and larger quantities of calcium during growth, especially large quantities during the last two months of pregnancy, and that the maternal blood shows a distinct tendency for its calcium content to decrease during the 8th and 9th months of pregnancy indicates the importance of a sufficiency of calcium in the diet of the mother during this time. Otherwise the calcium needs of the foetus may be supplied by withdrawing too large an amount from the tissues of the mother (*e.g.* bone, teeth), as it is evident from the figures in the above tables that the blood tends to maintain a fairly constant value in spite of the heavy demands made upon it. The serum of the blood of a certain number of cases has been examined for calcium where there have been continuous miscarriages or still-born infants, and where a variation might be expected. So far no very definite relationship seems to be shown. These results are given in Table V.

Number 14 gave a calcium content on one occasion a week after miscarriage of 1.1 mg.

The case of a haemophylliac was interesting. The calcium content of the serum per 10 cc. at the 7th month of pregnancy was 0.66 mg., at the 8th month 1.05 mg., and after being treated with calcium chloride for a month, at the 9th month was 1.12 mg. About nine months later the same case when not pregnant gave 0.83 mg. of calcium per 10 cc. of serum, and after treating with calcium chloride gave 0.94 mg. of calcium.

Table V.

Case	Characteristics of case	Calcium found	Percentage increase or decrease of calcium compared with average. (Calcium average 0.99 mg. per 10 cc. of serum.)	
1	Sterile	1.14	15.1	+
2	Five miscarriages	1.23	24.2	+
3	Still birth	1.09	10.1	+
4	Three miscarriages	1.08	9.09	+
5	Still birth	1.03	4.04	+
6	Twelve miscarriages	1.00	1.01	+
7	Six still-born	1.00	1.01	+
8	Several miscarriages	0.961	2.92	-
9	Several miscarriages	1.02	3.03	+
10	Several miscarriages	0.98	1.01	-
11	Four miscarriages and one still-born	0.996	0.6	-
12	Six miscarriages	0.918	7.2	-
13	Four premature births	0.88	11.11	-
14	Four miscarriages	0.74	25.2	-
15	Several miscarriages	0.561	43.3	-

Investigations have also been made into the question of the variation of the calcium content of the blood in connection with menstruation. These results have already been published [Widdows, 1922]. Since the calcium content of the blood of pregnant women is at the commencement of pregnancy slightly higher than that of non-pregnant women it was expected that there might be a rise in the calcium content at the beginning of menstruation. Bell [1908] had indicated that there was a rise just before menstruation followed by a very marked drop. Sherman, Gillett and Pope [1918] could show no distinct monthly cycle in the metabolism of nitrogen, phosphorus or calcium in the case of women. Although in two of the cases referred to in the *Lancet* of November 11th, 1922, there was a suggestion of a rhythmic rise and fall in the calcium content of the serum of the blood, the variations shown by the figures as a whole are not greater than those obtained in any normal series of estimations of blood calcium, and do not support the idea of a cyclic variation in the calcium content of the blood. The question of the relationship of the amount of calcium to the phosphorus in the blood has always been kept in view, and it was intended to take up that point this coming year, but de Wesselow [1922] has already examined his cases for this relationship and shows that the amount of phosphorus decreases in the later phases of pregnancy similarly to the calcium, but that during lactation there is a relatively high value for phosphorus in the plasma compared with that of the non-lactating female.

This work emphasises the perfect regulation of the human organism to the many and varied demands made upon it. The very great demand that undoubtedly at certain stages of its development is made by the foetus for calcium, is certainly met by a very perfectly balanced calcium metabolism, as at no time is the variation of the calcium content of the serum very marked. Even so in the later stages of pregnancy there does seem to be a distinct tendency for the calcium content of the blood to decrease. This indicates that at this time attention should be paid to the diet of the mother from the point

of view of its calcium content. During the last two months of pregnancy milk (on account of its high percentage of calcium) should be given the mother in a larger amount than that included in the average adult's diet and should be considered a necessary constituent of her diet.

I have to thank the Medical Research Council for the help that I have received during the present investigation.

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V. A METHOD FOR THE QUANTITATIVE ESTIMATION OF CHOLINE IN BLOOD.

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(Received January 1st, 1923.)

THE chemical methods at present available for the quantitative determination of choline are unsatisfactory. Those most commonly employed depend on the compounds formed with the heavy metals. But these are fairly soluble in water, in some cases to the extent of 25 %. The aurichloride is the least soluble, one part dissolving in 75 parts of water.

The biological method of testing the acetylated product upon the frog's heart suggested by Reid Hunt [1915] and later by Fühner [1916] can at present be regarded as affording only a rough estimation of the amount of choline.

The method of detecting the presence of choline in blood and cerebro-spinal fluid described by Allen [1904] seemed to promise the possibility of being developed into a quantitative process. Staněk [1905] described such a process in which he either estimated the nitrogen of the choline compound, or decomposed the periodide with cuprous chloride. The details of the second method were criticised by Kiesels.

The following method has been devised and tested, and it appears to be free of the difficulties of Staněk's procedure.

The principle is to extract the choline from the blood or tissues and to precipitate it with iodine as periodide, to wash off any free iodine and to decompose the periodide with dilute nitric acid, finally to extract the free iodine with chloroform and estimate it using a standard solution of sodium thiosulphate.

1 cc. N/20 thiosulphate sol. = 0.0007 g. choline HCl.

From 20 to 50 cc. of blood are drawn off into about four times this volume of absolute alcohol and well shaken. The containing vessel is allowed to stand for about 12 hours, after which time the contents are filtered into an evaporating basin. The precipitate is rubbed up and well washed with absolute alcohol and then is added to the filtrate. The filtrate is evaporated to a syrup and taken up in distilled water. The possibility of the extraction and decomposition of lecithin with the liberation of choline at this stage is considered later. The fluid is again evaporated to a syrup and the process repeated till it is alcohol-free. The solution is then transferred to a dialysing thimble of about 15 cc. capacity and dialysed for 24 hours into 200 cc. distilled water.

It is advisable to repeat this dialysis and to combine the resulting liquids. This is then evaporated to about 5 cc. and carefully washed into a beaker keeping the bulk as low as possible. 20 to 30 vols. of saturated iodine in potassium iodide solution are now added and the beaker set aside over night to allow the choline periodide to crystallise. The crystals are then filtered through a Gooch crucible on a layer of fine asbestos wool. They are washed repeatedly with ice-cold water until free from the precipitating solution. The Buchner flask is cleared out with cold water. The crucible is placed in position for filtering and the choline periodide is now ready for decomposition. This is accomplished by a mixture of 2 parts diluted nitric acid (1 part HNO_3 to 1 part water) with 1 part chloroform.

The above mixture is then shaken and added to the crucible. The periodide is decomposed by the nitric acid and the free iodine is taken up by the chloroform and carried into the flask by the suction. This is repeated several times until the asbestos is quite white. The crucible is now removed, more chloroform is added to the flask and well shaken in order to extract any iodine dissolved in the water.

The chloroform layer is then washed acid-free by decantation with repeated quantities of cold water.

The liberated iodine in the chloroform layer is now most conveniently titrated by $N/20$ sol. of sodium thiosulphate until the pink colour just disappears.

Choline hydrochloride added to normal blood:

A. Blood, 20 cc.; choline HCl, 4.9 mg.; cc. $N/20$ thiosulphate, 6.4; recovery = $6.4 \times 0.0007 = 4.48$ mg. = 91.5 %.

B. Blood, 20 cc.; choline HCl, 1.9 mg.; cc. $N/20$ thiosulphate, 2.55; recovery = 94 %.

C. Blood, 20 cc.; choline HCl, 17.0 mg.; cc. $N/20$ thiosulphate, 22.0; recovery = 90.6 %.

Tests on normal blood without the addition of choline:

Blood, 25 cc.; no choline HCl.

No precipitate of periodide was produced even on long standing.

The possibility of lecithin breaking down is small if the process is carried out with reasonable speed. Tests performed on blood to which no choline was added gave negative results. If there was any free choline present its concentration was below the limit of recovery by this process.

My thanks are due to Professor Noël Paton for his help and advice.

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VI. THE INORGANIC PHOSPHORUS CONTENT OF THE BLOOD OF NORMAL CHILDREN

PRELIMINARY COMMUNICATION

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(Received January 1st, 1923.)

ATTENTION has recently been directed by Howland and Kramer [1921] to the fact that the inorganic phosphorus content of the serum is lower in rachitic than in normal children. Hess has confirmed this finding [Hess and Unger, 1922], but states [Hess and Lundagen, 1922] that in some cases in which the presence of rickets was undoubted he found no reduction. He has further called attention to the fact that a slight seasonal variation of the amount of inorganic phosphorus in the blood of healthy children occurs, the highest figures being obtained in July, and the lowest in March. György [1922] and von Meysenbug [1922] have also published figures showing the reduction of inorganic phosphorus in the blood of rachitic infants. Furthermore, considerable attention is at present directed towards the question of the possibility of producing rickets in rats by a deficiency of phosphoric acid in the diet. It seemed desirable to repeat and extend these observations, and the prevalence of rickets in the slum districts of Glasgow from which our hospital patients are drawn has been taken advantage of to prosecute such a study. This investigation is still in progress.

One of the first essentials in undertaking an investigation like the present one is, of course, a knowledge of the amounts of inorganic phosphorus to be found, and the range of physiological variation occurring in the blood of healthy children. Table I shows the results of other investigators.

Table I.

Authors	Inorganic phosphorus as mg. per 100 cc.		
	Whole blood	Plasma	Serum
Howland and Kramer [1921]	—	—	4.5 to 6.8
Tisdall [1922]	—	—	4.6 to 6.4
György [1922]	5.2	—	—
Hess and Unger [1922]	4.0 to 4.8	—	—
Von Meysenbug [1922]... ..	—	—	4.1 to 5.8
McKelleps, Do Young and Bloor [1921] ...	—	1.2 to 4.4	—

The results agree fairly closely and indicate that the range of variation, if one excludes the very low figure of 1.2 given by McKelleps, De Young and Bloor, is 4.0 to 6.8 mg. per 100 cc.

In the course of a series of estimations of the phosphorus contents of the blood and serum for comparison with rachitic figures, one or two interesting points emerged and are therefore recorded in this preliminary report.

EXPERIMENTAL ERROR.

The method used throughout these experiments is that of Bell and Doisey [1920]. Marriott and Haessler's technique [1917], as well as that of Tisdall [1922] and that of Bloor [1918] take much longer to complete without giving more accurate results.

Myers and Shevsky [1921] have recently criticised the method of Bell and Doisey and state that more accurate results are obtained when the standard contains not over 0.25 % more phosphorus than the specimen to be examined. They further observed that blood sometimes requires more of the molybdic acid and quinol solution than does serum. For this reason the standards used throughout this investigation are as far as possible made up to contain a quantity of phosphorus similar to that of the blood sample to be tested, and for whole blood double the quantities of molybdic acid and quinol advocated by Bell and Doisey are used.

In order to gain an estimate of the experimental error involved in the use of the method 14 consecutive estimations on the same blood sample were carried out. In each case 5 or 10 cc. of lysed diluted dog's blood were separately treated and subjected to colorimetric comparison with a freshly made up standard. The calculated values of phosphorus per 100 cc. of the unknown are given in Table II.

Table II.

	Quantity of diluted blood used	Estimated values of P per 100 cc. of diluted blood
	cc.	mg.
1	10	1.7
2	10	1.9
3	10	2.0
4	5	1.8
5	10	2.1
6	10	2.0
7	5	1.8
8	5	1.8
9	10	2.0
10	10	1.9
11	10	1.9
12	5	1.9
13	10	2.1
14	10	1.7
		<hr/>
		Average 1.9 +0.2 -0.2

From the series of figures it is estimated that the error is plus or minus 0.2 when the average is 1.9, or roughly 10 %. This is therefore taken as the margin of experimental error.

THE DISTRIBUTION OF INORGANIC PHOSPHORUS IN THE BLOOD.

An interpretation of the published results of inorganic phosphorus estimations in normal children is, as in the case of calcium, rendered somewhat difficult in view of the fact that in some cases serum, in others plasma, and in still others whole blood has been used, since our knowledge of the distribution of the inorganic salts is at best very incomplete.

Howland and Kramer [1921] pointed out that a definite increase in the phosphorus of the serum results if the latter is allowed to stand in contact with the clot at room temperature for any length of time. This introduces a grave source of error in serum estimations and renders any investigation into the distribution of the inorganic phosphorus between corpuscles and plasma particularly difficult. The error which may result from this change is illustrated by two different series of blood and serum estimations in normal children. In every case the whole blood was immediately shaken up in 15 cc. of distilled water, 5 cc. of trichloroacetic acid added and the flask vigorously shaken and allowed to stand ten minutes before filtering off the protein. In the first series of cases no time limit was set for the separation of the serum from the clot though all were ready for filtration within three hours of the collection of the blood. The blood samples of the second series were subjected to 40 minutes' centrifugalisation immediately after venipuncture and the serum was then quickly poured off. The results are collected in Tables III and IV.

Table III. Series 1.

Blood P estimated immediately and serum P estimated after standing from 1½ to 3 hours in contact with the clot.

Number	Age (years)	Sex	Inorganic P of whole blood mg. per 100 cc.	Inorganic P of serum mg. per 100 cc.
1	8	Male	5.1	6.1
2	8	"	5.4	7.2
3	7	"	—	6.0
4	9	"	5.1	5.5
5	10	"	4.8	6.0
6	8	"	4.0	4.8
7	12	"	—	6.8
8	13	"	4.8	6.5
			Average 4.86	Average 6.1
			Range of variation 4.0 to 5.4	Range of variation 4.8 to 7.2

Table IV. Series 2.

Inorganic P of whole blood estimated immediately. Serum in every case separated from clot within 40 minutes of venipuncture.

Number	Age (years)	Sex	Inorganic P of whole blood	Inorganic P of serum
1	3	Female	—	4.6
2	5	Male	4.2	4.2
3	6	"	4.0	4.2
4	6	"	4.8	5.1
5	7	"	4.2	4.4
6	9	"	5.1	5.4
7	10	"	4.8	4.7
8	11	"	5.1	5.1
			Average 4.6	Average 4.7
			Range of variation 4.0 to 5.1	Range of variation 4.2 to 5.4

It is quite evident that all the serum results of Series 1 are higher than those of Series 2 and since the estimations were carried out within a few days of each other and the same standard solutions employed, one can only conclude that the difference must be ascribed to the greater length of time taken to separate the serum in the first series.

The fact that the serum phosphorus is so variable, therefore, seems to render it unsuitable for comparative estimations, particularly since we know little of the changes which may be taking place during the 30 minutes which must of necessity elapse before any quantity of serum can be separated off.

Denis and von Meysenbug [1922] have criticised the use of plasma on the ground that they found citrate and oxalate to be a source of considerable error in the use of the Bell-Doisey method.

From a consideration of these points it appears that whole blood is most suitable for use in estimating the inorganic phosphorus, for though a definite increase also occurs in the lysed blood on standing each specimen can be subjected to exactly the same procedure immediately it is withdrawn from the vein.

A study of the figures given in Table IV leaves no doubt that the whole blood if used at once gives exactly the same results as serum which has been separated from the clot within 40 minutes. This leads one to suppose that the corpuscles contain exactly the same amount of inorganic phosphorus as the serum, or that, if the corpuscles are richer in this element than the serum or plasmas, as suggested by Bloor [1918], a very rapid change in the distribution of the blood phosphates must take place during the process of clotting. As a standard for comparison with the rachitic figures the collected results for the whole blood of all the normal children available are grouped in Table V.

Table V.

Number	Date 1922	Sex	Age	State of health and nutrition	Inorganic P of whole blood in mg. per 100 cc.
1	July	Male	9 mths.	Normal	5.2
2	"	Female	5 yrs.	"	4.2
3	"	Male	6 "	"	4.0
4	"	"	6 "	"	4.8
5	"	"	7 "	"	4.2
6	"	"	8 "	"	5.1
7	"	"	8 "	"	4.0
8	Aug.	"	9 mths.	"	6.6
9	"	"	9 yrs.	"	5.1
10	"	"	9 "	"	5.1
11	"	"	10 "	"	4.8
12	"	"	10 "	"	4.8
13	"	"	11 "	"	5.1
14	"	"	12 "	"	5.3
15	"	"	13 "	"	4.8
16	Oct.	"	15 mths.	"	4.1
17	"	"	14 "	"	4.8
18	Nov.	"	8 "	"	5.0
19	"	"	8 "	"	6.6
20	"	"	14 "	"	6.2
21	Dec.	"	3 "	"	5.5
22	"	Female	2½ yrs.	(Convalescent pneumonia)	4.0
23	"	Male	3 mths.	Normal	4.7

Average = 4.9

Range of variation 4.0 to 6.6

The results in Table V show that the average normal phosphorus content of the blood in children is 4.9 mg. per 100 cc. The range of variation allowed for by a probable experimental error of 10 % is 4.4 to 5.4, whereas the actual range is 4.0 to 6.6. The variation in the normal amount of inorganic phosphorus in the blood is therefore not entirely due to experimental error and no figures between 4.0 and 6.6 mg. per 100 cc. can be regarded as abnormal. Children under two years undoubtedly give slightly higher results than those over three years, while in two adults whose blood has been examined the results are still lower than those obtained from the older children. That is to say, the inorganic phosphorus of the blood appears to fall with increase in age, as pointed out by Howland and Kramer, but the seasonal variation described by Hess and Lundagen [1922] has not been apparent from the figures so far available.

EFFECT OF FEEDING ON THE NORMAL BLOOD PHOSPHORUS CONTENT.

In the case of the younger children some were breast fed and some bottle fed on different diets, but there appeared to be no connection between the type of feeding and the phosphorus values obtained. The question as to whether an increase in the inorganic phosphorus may result from the recent consumption of phosphorus-rich diet is, however, one which must be raised, since it seems not improbable that the slight variations obtained may be due to differences in the stage of digestion. For the purpose of throwing light on this problem the following experiment is recorded. In the course of a parathyroidectomy investigation carried out by Professor Noël Paton and Mr Watson, whom I have to thank for permission to publish this result, the latter took from the jugular vein of a dog samples of blood, (a) after a period of fasting, and (b) at increasing intervals after the ingestion of a quantity of lean meat.

The results of the experiment are tabulated below.

Table VI.

		Inorganic P of whole blood in mg. per 100 cc.				
Time		Remarks				
Exp. 1	9.30 a.m.	After 15 hours' fasting	2.8 %
	11.30 a.m.	" 17 "	"	2.9
	2.30 p.m.	" 20 "	"	3.0
	6.30 p.m.	" 24 "	"	2.8
Exp. 2	1 p.m.	No food for 6 hours. Given 200 g. lean meat	—
	2 p.m.	1 hour after food	2.97
	3 p.m.	2 hrs. " "	3.17
	4 p.m.	3 hours " "	2.97

The above figures show that the amount of inorganic phosphorus in the blood is uninfluenced by fasting for 24 hours and by the recent ingestion of a quantity of food rich in phosphorus.

CONCLUSIONS.

1. The range of experimental error in the Bell-Doisey method of estimating the inorganic phosphorus of the whole blood was found to be not more than plus or minus 10 %.

2. The inorganic P content of the whole blood of 24 normal children between 3 months and 13 years of age ranges between 4.0 and 6.6 mg. per 100 cc. Average 4.9.

3. Blood and serum give the same results provided the blood protein is precipitated immediately and the serum is separated from the clot within 40 minutes.

4. The use of serum which has been allowed to stand in contact with the clot for longer periods may be a source of considerable error. It is therefore better to use the whole blood.

5. Twenty-four hours' fasting and the recent ingestion of a diet rich in phosphorus failed to produce any demonstrable variation in the inorganic blood phosphorus content of a normal dog.

I desire to express my thanks for advice and criticism to Professor Noël Paton and Dr Leonard Findlay, under whose direction this investigation was carried out. All expenses connected with the work have been defrayed by the Medical Research Council.

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VII. SEASONAL VARIATIONS IN THE DISSOLVED OXYGEN CONTENT OF THE WATER OF THE THAMES ESTUARY.

WITH SPECIAL REFERENCE TO THE PHENOMENON OF SUPERSATURATION

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(Received January 1st, 1923.)

THE observations recorded below were made as part of a general investigation of the River Thames undertaken primarily from a public health point of view and the phenomena have only been investigated so far as was compatible with the main objects of the scheme. Casual examination of the water of the estuary of the Thames in the summer of 1914, in the course of which determinations of dissolved oxygen and chlorions were made, yielded several results in which the proportion of dissolved oxygen was much greater than was required for saturation with atmospheric oxygen of water of the observed salinity. Systematic observations made in subsequent years showed that supersaturation with dissolved oxygen occurred to a greater or less extent in each spring and in a less systematic manner later in the year.

Until recently we have found no direct references in scientific literature to this phenomenon of supersaturation in sea water, although it has been observed and its cause properly assigned in fresh water. One of us has pointed out [Coste, 1917] that "supersaturation—at any rate with oxygen—is by no means rare." It is sometimes to be attributed to "rapid variations of temperature," but "more often to its excretion from vegetation as a residual product of photosynthesis." In this paper it is noted that Morren (1843), who investigated the gaseous content of the surface water near St Malo, found that the oxygen content of sea water was greatest and the carbon dioxide least in bright weather. It should also be noted that the New York Harbour Board investigators recorded several cases where the volume of dissolved oxygen found was greater than that required for saturation, although they refrained from recording a greater percentage saturation than 100.

Recently Dr J. H. Orton, with whom we have been in correspondence, called our attention to a paper by Schulz [1922] in which he records and discusses cases of supersaturation with dissolved oxygen in the North Sea. Schulz refers to an earlier paper by Gehrke [1916] in which supersaturations up to 16 % are recorded.





The maxima for May and June are very much greater than in any other months, and the sequence of maxima shows that there is a growing tendency towards this maximum, with a progressive falling off from it towards the end of the year. The years 1916 and 1918 are outstanding as years of extremely high supersaturation. As to places, supersaturation was on the whole more pronounced in the neighbourhood of the West Oaze Buoy than in any other place and least frequent and less in amount at the upper stations, the Mucking and Chapman Lights and off Southend, although in 1916, very high figures were observed in May—above 50 % excess of dissolved oxygen—at all places from the Mucking Light to the Knob Buoy.

DISCUSSION OF RESULTS.

In the first place we would emphasise the fact that the supersaturation with dissolved oxygen is real. In the early stages of this work we realised that 2 % above or below saturation might be allowed for experimental error + the uncertainty of the standard figure adopted, and that high barometric pressure might at times cause the standard figure based on a pressure of 760 mm. to be about 2 % below the actual figure appropriate to such high pressure. If all these errors by an unfortunate combination were of the same sign, they might cause a figure of 104 % to be obtained when the true figure was only 100, but they would not account for the much higher figures frequently obtained. If water, say from polar regions, could somehow be raised from 0° to 15° without losing dissolved oxygen, this would raise 100 % saturation to about 140, but such supersaturation of oxygen would be accompanied by a similar supersaturation of "nitrogen" ($N + A$).

It should be mentioned, however, that while this range of possible error must be recognised, the lower degrees of supersaturation conformed to the rule which characterised the higher ranges. They were uniform—with minor variations—for each point of observation and they represented a definite rise through a period often of several days, as contrasted with prolonged returns of figures at or slightly below the saturation point. While admitting them with caution and subject to possible errors which have not been eliminated, it would we think be a mistake to dismiss them as due entirely to the possible sources of error mentioned. It is desired to note in passing, merely, that our observations suggest that variations in the amount of dissolved oxygen in estuarial waters above or below the saturation point are of not infrequent occurrence and are observed under conditions which are not likely to be explained by the periodic photosynthesis associated with the higher figures of supersaturation. Similar small divergencies from saturation have been noticed in the North Sea by Schultz.

Any explanation of the phenomenon of natural supersaturation based on the assumption of retention of oxygen in water recently subject to a rise of temperature such as might occur in the case of polar currents reaching the

North Sea fails, as will be seen from the following observations, to account for all the facts. Using Winkler's gasometric method, E. T. Butler and F. J. East in 1917 found that the proportion of nitrogen in water supersaturated with oxygen was normal, *i.e.* was approximately equivalent to that required for nitrogen saturation at the observed temperature. One result where the proportion of oxygen was rather less than 100 % is included in the table.

Table II. *Showing the percentage saturation of estuary water with oxygen and nitrogen in June, 1917.*

Place	Date	Temp.	Cl.	Dissolved gases cc. per litre		Percentage saturation	
				Oxygen	Nitrogen	Oxygen	Nitrogen
West Oaze	June 12	17.7°	1860	8.1	11.1	134	104
Nore	" 14	18.0°	1845	7.9	11.0	150	104
"	" 15	18.6°	1860	8.5	10.7	162	102
"	" 18	19.7°	1870	7.44	10.7	145	104
Chapman	" 19	20.3°	1505	4.89	11.0	90	104
West Oaze	" 22	19.10°	1875	6.13	10.5	119	101

The one obvious explanation of supersaturation with oxygen only in spring is to be found in the normal vernal phenomena. An increased duration of daylight and greater penetrating power of the sun's rays lead to the rapid growth of the varied algal flora of the sea [cf. Moore, 1921] and thus to photosynthesis. The rising temperature of the water increases the velocity of photosynthesis as it does of other chemical reactions. This point has been investigated by Osterhout and Haas [1919] who found the temperature coefficient to be about 1.81 per 10° between 17° and 27°.

That water should become and remain appreciably supersaturated with a gas whilst it is in free contact with air is at first startling, and as we have indicated, we were at pains to satisfy ourselves of the reality of the phenomenon.

It is obvious that, given a source of pure oxygen (such as a growing plant) in water, the water around this source will take up the oxygen very rapidly if it is generated all over (or within) the surface of contact between water and plant without the formation of a limited interface which would occur if bubbles were generated. It would in fact appear that there will be no tendency to form bubbles until the concentration of dissolved oxygen is nearly five times as much as in water in equilibrium with air, since it is pure oxygen and not air which is evolved by the plant. Hence very little loss by reason of non-absorption by the water of oxygen generated is likely to occur. Loss may, however, occur in two ways: (1) by combination of the oxygen with oxidisable matter either with or without the aid of living organisms; (2) by escape into the air at the interface of air and water, following an upward diffusion of oxygen in solution. The amount of oxygen which can be liberated by photosynthesis is limited on the one hand by the relative abundance of chlorophyllous organisms and, on the other, by the available carbonic acid. Prideaux, Moore

and Herdman [1915] have shown that in sea water the removal of CO_2 from bicarbonate by a green algal growth can go on until the transformation



is complete, but not beyond this point. This represents a p_{H} of about 9.1.

Sea water contains about 2.4 milliequivalents per litre of "uncompensated" base mostly as bicarbonate. If it be considered as all present as bicarbonate, then 2.4 milliequivalents of CO_2 are available for liberation and transformation into organic compounds and oxygen. The evolution of oxygen has been shown by Schulz [1922] to be equivalent within small limits to the CO_2 transformed. It is therefore possible in one litre of sea water to liberate 1.2 millimols. of oxygen or about 27 cc. of oxygen at N.T.P. This is from four to five times the amount required to saturate sea water in equilibrium with air. It is, in fact, almost enough to saturate it if under an atmosphere of pure oxygen. Further, the sea water is usually approximately saturated (with respect to the 21 % of oxygen in air) already, and also an increase of the alkalinity of sea water will cause it to take up more CO_2 (from which more oxygen can be liberated) from the air. There is, therefore, no difficulty in accounting for the liberation of oxygen in the required amount, given the presence of quick-growing organisms. It is reasonable, however, to expect that this liberation of oxygen should be accompanied by a decreased hydrion concentration.

Casual determinations of hydrion concentration during 1920 showed that there was a seasonal alteration of the kind expected (Table III). The results expressed as p_{H} , *i.e.* Briggsian log. of reciprocal of hydrion concentration—were as follows for sets of samples in March, April, May and June, 1920.

Table III. *Hydrion concentration of river water. Spring and early summer, 1920. (Expressed as p_{H} .)*

	Mar. 26	Apr. 22	May 5	June 7	June 9
Erith	7.78	7.78	about	7.76	7.69
Greenhithe	7.78	7.76	7.76	7.78	7.69
Gravesend	7.75	7.78	or less	7.78	7.69
Mucking Light	7.88	7.78	7.78	8.08	8.08
Chapman Light	—	7.78	7.88	8.21	8.20
Southend	7.88	7.88	8.20	8.41	8.31
Nore Light	7.94	7.88	8.20	8.41	8.31
West Oaze Buoy	7.94	7.94	8.20	8.41	8.30
Knock John Buoy	7.94	7.94	8.20	8.41	8.31
Edinburgh Light	7.94	7.94	8.20	8.31	8.20

It will be noted how the real alkalinity, as shown by hydrion concentration, on the whole increased at stations from the Chapman Light downward from March to June.

From June 1921, a regular series of observations was made, using Prideaux's method of double titration [1919]. The results of these are set out for one typical place in the estuary—the Knock John Buoy in Fig. 2, which shows the variation in p_{H} (hydrion concentration) for the period in question. It will be seen that during a great part of the period—from July 1921 to April 1922—

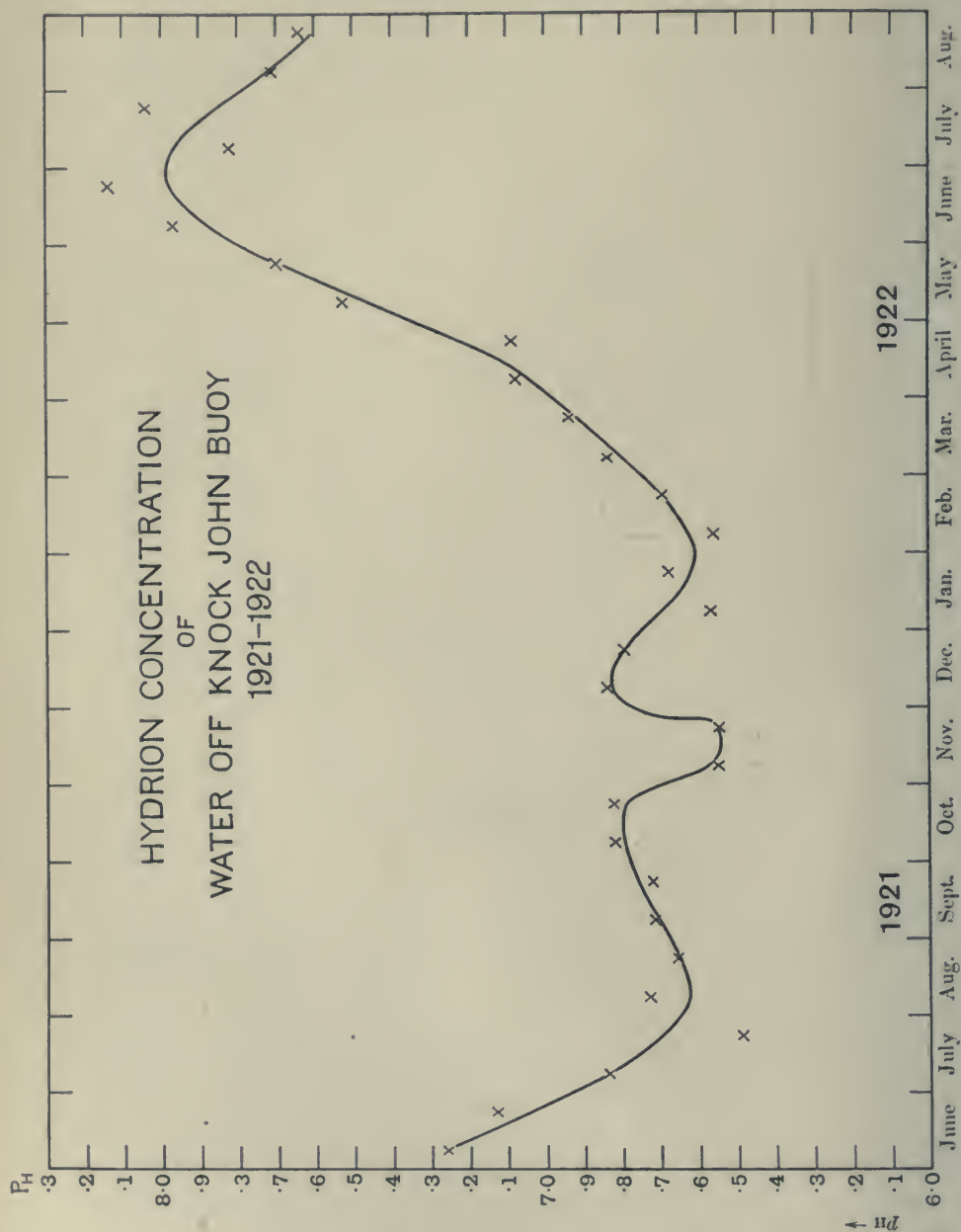


Fig. 2.

the water was acid, becoming from April 1922 to June 1922 more and more alkaline, with some indications of a falling off from June. Further the water was alkaline at the beginning of the period—June 1921.

These figures agree with the argument just developed that marked alkalinity and supersaturation should go on together, or rather that if supersaturation is observed, it should be at periods of high alkalinity (low hydron), the latter being a more lasting record of photosynthesis than supersaturation.

There is some positive evidence of an increase of microscopical green organisms in the estuary water towards summer. It was obviously impossible to include in an investigation undertaken from a public health point of view, a full examination of microplankton but a regular microscopical examination of the sediment from the water was begun in July 1921 and the results returned, in respect of light green growths, dark (blue) green growths and diatoms, as trace (1), few (2), many (3), chiefly (4). The numbers attached were used to represent approximate orders of magnitude, and as nearly all the observations were made by one worker (Mr E. T. Butler) they should be sufficient to indicate a general increase or decrease of green organisms. Fig. 3 shows in graphic form the seasonal variation which, on the whole, follows the course suggested in this discussion.

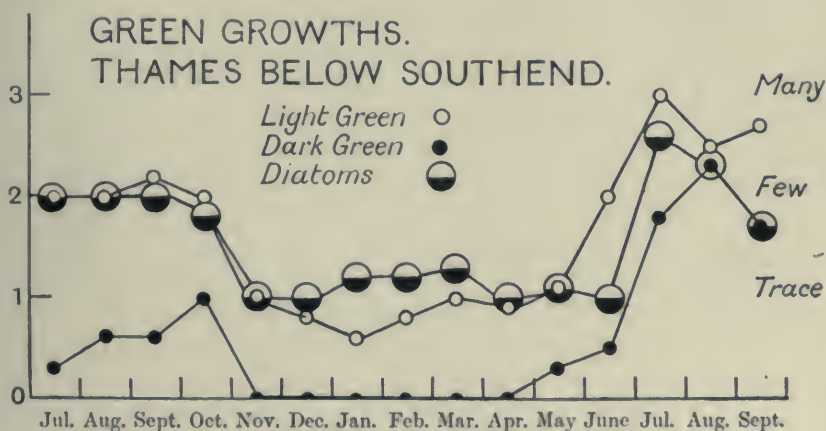


Fig. 3.

Sir W. A. Herdman [1922] has discussed "The distribution of the plankton as a whole and of its various constituents throughout the year" in the Irish sea. He says "The spring maximum of phyto-plankton starting in March, when the sea has still a low temperature and increasing to a climax in April, May or June; the diatom minimum at the height of summer in July or August; the secondary lesser maximum in autumn (generally September or October), very variable both in extent and in constituent organisms; and finally, the winter minimum have been recorded for every year and need no further demonstration."

The interaction between a gas (such as air) and a liquid in which it is soluble (such as water) is only a special case of heterogeneous reaction. It has been discussed generally by one of us [Coste, 1917] and the side of the equilibrium where the liquid is supersaturated with the gas was investigated by C. Bohr for solutions of carbonic acid [1899]. The case of unsaturation has recently been carefully investigated by Adeney and Becker [1919, 1, 2]. The general equation for the course of this action when the liquid is of uniform composition below the surface

$$\frac{C - x_t}{C - x_0} = e^{-kt} \text{ whence } \frac{dx}{dt} = k(C - x)$$

where C = the concentration corresponding to saturation, x_0 the initial concentration, x_t the concentration at any period of time t from the beginning of the action and k a constant which is really the evasion constant (a multiple of the *coefficient of evasion* of Bohr) is applicable to cases of supersaturation as well as to unsaturation, the only difference being that $C - x$ has a negative value both above and below the line on the left of the equation, as also has dx . Since this general equation implies that the rate of approach to saturation is, all other conditions being similar, proportional to the difference from saturation, it follows that around saturation, towards which the value converges, the rate of exchange becomes very slow, whether the liquid is supersaturated or unsaturated with the gas. The value of k will be small if the motion of water or air is slight, but it will increase rapidly if the molecules at the interface are rapidly changed, or if the area is enlarged. Hence still weather, when the motion of air and water is small, will tend to preserve supersaturation, whilst rough weather, when both air and water are in rapid motion, and waves, spray and air bubbles increase the interfacial area, will tend to bring about mere saturation, that is, stable equilibrium more rapidly. The effect of windy weather in increasing the dissolved oxygen in unsaturated water has been observed by R. H. Gould [1922] in the water of the Arthur Kill, New York Harbour. Our own observations on supersaturated water conform to those of these observers. Supersaturations in the estuary disappear rapidly under agitated conditions of the water, due to wind, and the comparatively low supersaturations observed in the years 1919–1922 are not improbably due to the coincidence of disturbed weather with the period of highest photosynthetic activity.

In still weather, the vertical movement of water due to convection by reason of the cooling and concentration of salts in the surface layer by evaporation is at a minimum and exchanges of dissolved gas (oxygen produced under the surface) between layers of water in different vertical planes will proceed more nearly by pure diffusion according to Fick's law [1855] causing a gradient of concentration, and less by the more efficient process of mixing. The considerable depth of the sea renders the formation of a gradient more easily possible.

Two experiments made by us illustrate the slowness of escape of oxygen from still water and the accelerating effect of agitation.

1. A large Winchester bottle was filled with tap water and inverted over water so that about $\frac{1}{2}$ litre of oxygen could replace some of the water. The bottle was stoppered and shaken repeatedly, and then left inverted with a water seal all night. Next day the water was siphoned off into another bottle which was filled to the neck. At intervals, samples were removed with suitable precautions for the determination of dissolved oxygen. The first three samples were taken within one hour and as the surface exposed was small, the loss of dissolved oxygen, although distinct, was small. The water level then reached the shoulder of the bottle and the surface exposed after this was practically constant. The results are shown in Table IV.

Table IV.

Time (hours)	0	$\frac{1}{2}$	1	3	47		51	52	144
Temperature	16.8	16.85	17.0	17.3	17.8	17.8 ¹	18.9	19.4	16.0
Dissolved oxygen cc. per litre	24.4	24.4	24.3	24.2	11.44	10.8	11.12	10.8	6.36
% saturation	351	351	355	356	173	151	171	168	92.0

¹ Taken at top of water. All others siphoned from bottom. Value of k calculated from 47 hours = 0.026.

These results show how slowly still water parts with an excess of oxygen and those for 47 hours illustrate the working of Fick's law. The last result is to be explained by temperature changes. Barometric variations were considered in calculating the saturation values.

2. Similarly prepared water was placed in a cylindrical glass jar, exposing a surface of 95 cm.² and stirred with a spiral glass stirrer at such a rate as not to break the surface. The results are stated in Table V.

Table V.

Time (hours)	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4
Temperature	16.6	16.7	16.6	16.6	16.6	16.8	17.1	17.4
Dissolved oxygen cc. per litre	23.34	21.56	19.38	18.2	16.95	13.38	10.05	8.85
% saturation	338	312	281	264	253	195	148	127.5

Value of k about 0.5.

The effect of stirring in accelerating the exchanges between air and water is very marked, the value of the evasion constant being increased about 20 times [cf. Becker, 1921]. The experiment illustrates the effect of rough weather in rapidly dissipating supersaturation of sea water with oxygen or indeed with any dissolved gas.

It may be remarked that whereas the water of such rivers as the Thames or Seine is always much supersaturated with carbonic acid, that is, with calcium bicarbonate—in sea water which is much more agitated and mixed with the air the equilibrium is more closely approached [cf. Prideaux, 1919].

If what we have observed in the Thames estuary is true generally, not only of the seas around our coasts but also of the oceans beyond, the phenomenon is of more than academic interest. The vernal supersaturation of sea water, which from the nature of our observations was necessarily striking, is

only an index of a process which cannot fail to have results in much wider and unexplored fields. The seas at these times are a great natural factory where free oxygen is produced on a scale which, having regard to the relative areas of sea and land, may conceivably exceed that of land vegetation. The oxygen of supersaturation is but a proportion and probably a small one, of the volume set free by marine photosynthesis. It is tempting to speculate on the part played by these gases in the contribution to the salubrity of sea air. The tidal convection of oxygen during periods of supersaturation is no negligible datum among the purifying factors of polluted estuarial waters. The seas breaking on the shores at these times are liberating their surcharge of oxygen in its most active state and it is seen that not the chemist or the marine biologist alone is interested in a phenomenon which has for its theatre of operations the vast tracts of oceans and the littorals of continents.

It has always been realised that the sea acts as a great stabiliser of the CO_2 content of the air by reason of the bicarbonate-carbonate equilibrium. It appears that it is equally efficacious in the positive work of keeping up by continual contributions a steady proportion of oxygen in the air.

We have mentioned various co-workers in the body of the paper. We wish particularly to acknowledge the work of our colleagues Messrs E. R. Andrews, J. W. H. Biggs and E. T. Butler, the latter of whom carried out most of the work afloat. They have all, often under circumstances of difficulty, discomfort, and even danger, in a variety of ways, contributed to the elucidation of the points now set forth. In the conduct of the observations their work has been indispensable, and the accumulated data essential to an adequate survey of the phenomenon are a tribute to the assiduity of their co-operation.

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VIII. THE EFFECT OF CARBON DIOXIDE AND ACETIC ACID ON THE OSMOTIC PRESSURE OF HAEMOGLOBIN.

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THE work of Martin [1896], Reid [1905], Hüfner and Gansser [1907], and Roaf [1910] has demonstrated that solutions of haemoglobin, as free as possible from electrolytes, have a measurable osmotic pressure. Measurements of osmotic pressure, therefore, offer a means of studying various changes in the physical and chemical state of haemoglobin in solution.

The purity of preparations studied in this way is, of course, of fundamental importance. Since haemoglobin is believed to exist in the blood as a sodium salt, there is the possibility that it may be isolated as the sodium salt rather than as the free acid. In order to remove any base which may be present, the solutions can be dialysed against water saturated with CO_2 , as used by Bayliss [1911] in connection with his experiments on Congo-red. On carrying out such a procedure in an osmometer, preparatory to certain experiments, the osmotic pressure was observed to rise to a height several times greater than its original value when distilled water alone constituted the outer fluid. When the CO_2 solution was replaced by water the pressure fell to its former value. The present paper records a study of these changes, and similar changes caused by acetic acid.

There are, as far as I know, no observations recorded in the literature on the effect of CO_2 on the osmotic pressure of haemoglobin. Roaf [1910] reports experiments using laked corpuscles or a solution of once-crystallised, dried haemoglobin (to which alkali had been added to get it into solution) on the inside of the osmometer and acetic or hydrochloric acid as the outer fluid. In most cases when the acid used was strong enough to cause a rise in the osmotic pressure, the haemoglobin was changed to acid haematin, and the resulting increase in osmotic pressure must have been due, at least in part, to the increased number of osmotically active particles caused by the breaking up of the haemoglobin molecule. In two experiments (the only ones at all comparable to those reported below) methaemoglobin resulted from the treatment, and slight changes in osmotic pressure were observed. 0.001 N HCl gave an increase from 17.5 mm. (the control value against water) to 24.9 mm.

Hg and 0.005 *N* acetic acid gave an increase from 7.5 mm. to 11.2 mm. Hg. In neither case was the increase greater than 50 %, while in the experiments to be reported, a much greater rise in osmotic pressure was observed. No attempt was made to see whether or not the effect due to acids could be reversed by the substitution of water. Roaf concludes, on slight evidence [1910, p. 85] "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance, sodium and "haemoglobyl" ions."

Preparation of haemoglobin.

The haemoglobin¹ was prepared according to the method described by Dudley and Evans [1921]. Well-washed corpuscles were laked by dialysing against distilled water in a collodion sac connected with a mercury manometer (see Hartridge [1917] for an earlier use of this method of laking corpuscles, to get a very concentrated solution of haemoglobin). Oxygen was bubbled through the thick haemoglobin solution after the stroma was removed by centrifuging. The crystals of oxyhaemoglobin which were formed in large amount by this procedure, were centrifuged from the mother-liquor and washed into a flask with one or two volumes of distilled water. By evacuating the flask, the oxyhaemoglobin was changed to the more soluble, reduced haemoglobin and the crystals passed into solution. Oxygen bubbled through this concentrated solution caused a recrystallisation of oxyhaemoglobin. Violent agitation of the flask when the oxygen is being bubbled through often helps to start crystallisation. This process may be repeated several times, though it is only fair to say that it becomes increasingly difficult to bring about this crystallisation. Perhaps a certain small percentage of inorganic salt is necessary for crystallisation, and this percentage may become reduced to a minimum as the purification continues. In these experiments, the haemoglobin preparations were always crystallised three, and usually four, times.

Great care was taken to prevent decomposition. The corpuscles were kept as cold as possible from the time of their shedding until the recrystallised haemoglobin was put into the osmometers (7–10 days). The NaCl used to wash the corpuscles was ice cold and the dialysis of the corpuscles, which took several days, was carried out in an ice box, at 4 to 7°. In the earlier experiments, the first crystallisation was brought about by the use of a minimal quantity of ice-cold alcohol added to corpuscles laked with an equal volume of water (the stroma removed, as in the other method, by centrifuging). The subsequent recrystallisation was carried out as given above. An experiment described on a subsequent page of this paper, shows that samples prepared

¹ The haemoglobin used in these experiments was prepared from the corpuscles of oxalated horse blood, obtained, in England, through the kindness of Dr R. A. O'Brien of the Wellcome Physiological Laboratories, and in the United States, through the kindness of Dr John A. Murphy of the Mulford Biological Laboratories.

in the two ways give the same osmotic pressure readings. On theoretical grounds, at any rate, the method of Dudley and Evans is certainly preferable, for alcohol, which may denature the protein, is not used.

Unfortunately, there is no very satisfactory way of determining the purity of a sample of haemoglobin. To test how far the recrystallisation removed the electrolytes from the haemoglobin solution, the conductivity of the mother-liquor (found to contain about 1 % haemoglobin) was tested after each crystallisation. For instance, in one preparation, the conductivity, determined roughly at room temperature (9.5°) was:

Mother-liquor above 1st crop of crystals	1.7×10^{-3}	mhos
" " 2nd " "	9.5×10^{-4}	"
" " 3rd " "	2.4×10^{-4}	"
" " 4th " "	6.0×10^{-5}	"
A 13.8 % solution of haemoglobin from the 4th crystals			2.5×10^{-5}	"

Another preparation (1.96 % solution) had a conductivity of 3.9×10^{-5} mhos at 18° . This figure is practically identical with that given by Gamgee [1902] for purified haemoglobin. He gives 3.25×10^{-5} for a 2.24 % solution at 18° . The conductivity of the laboratory distilled water, not free from CO_2 at 18° , was 4.25×10^{-6} .

If a saturated solution of haemoglobin is made up quickly, by adding distilled water to oxyhaemoglobin crystals, it is seldom stronger than 2 %. But if the packed haemoglobin crystals are allowed to stand, after removing the supernatant liquid, they will go into solution, sometimes not for several days, sometimes in the course of a few hours, even when kept on ice, giving a solution of 12 to 14 %. This solution gives a definite oxyhaemoglobin spectrum, but as it is difficult, spectroscopically, to distinguish reduced haemoglobin when a small amount of oxyhaemoglobin is present, it is not known how far reduction plays a part in this re-solution. Such a solution after several weeks' standing gives a definite spectrum of reduced haemoglobin. By dialysing corpuscles in collodion sacs against water, Hartridge [1917] was able to obtain solutions of haemoglobin as strong as 48 %. In speaking of the change in colour on dialysing, he says, p. 254: "This is due to the laking of the blood, and not to reduction of the haemoglobin, for if a thin film of the haemoglobin solution is removed from the dialysers it is a bright red in colour and analysis in Barcroft's apparatus shows that it is fully oxygenated." Further study is needed on this point. Both weak and strong solutions were used in these experiments.

Apparatus.

Osmometers of two types were used. Those which will be referred to as Type A were Moore and Roaf osmometers [1907], as modified by Bayliss [1909], with the exception that no precautions were taken to exclude the CO_2 of the air from the solutions in the osmometer. Type B osmometers were made of collodion sacs, fitted with U-tube mercury manometers of 1 mm. bore,

and suspended in bottles (about 150 cc. capacity) containing the outer fluid (Fig. 1). In order to use the minimum of solution (a large yield of haemoglobin does not result after so many crystallisations) and quickly to bring about an equilibrium between the inner contents of the osmometer and the outer fluid, use was made of a glass float which occupied almost the entire space within the collodion sac. Before sealing off the test-tube from which the float was made, mercury was admitted into the tube so that the float, when placed in the sac filled with haemoglobin solution, would slowly sink to the bottom of the sac. This prevented pressure on the bottom of the collodion sac and kept it from floating on the surface of the fluid and interfering with the opening into the manometer. Only 5 cc. of haemoglobin was then needed to fill the osmometer and there was only a thin film of solution between the glass float and the wall of the collodion sac, thus facilitating diffusion by doing away with the central core of liquid. Another advantage is that it is possible to see through such a thin film of solution, and note the appearance of any precipitate, change of colour, etc., in the solution in the osmometer. 10 % Anthony's negative cotton in equal parts of alcohol and ether was used to prepare the sacs.

Thymol and toluene were both found unsatisfactory for use in the osmometers or perfusing fluids, because after a time they caused precipitation of the haemoglobin. Haemoglobin solutions, however, used without any preservative did not undergo bacterial decomposition to any noticeable extent, even though kept for several weeks in the osmometers. Experiments were not continued any longer than necessary (usually less than two weeks) and in two cases osmometers were kept in an ice box at 4° throughout an experiment.

Briefly, the experiments were carried out as follows. The manometer tube was filled with haemoglobin above the mercury on the side to be connected with the collodion sac. The manometer carried a cork stopper which fitted into the reagent bottle containing the outer fluid of the osmometer. A float was placed in the collodion sac which was then filled with haemoglobin solution. A rubber stopper (with a hole for the manometer) was inserted securely, a rubber band placed around the neck of the sac and the joint covered with collodion. The manometer was then carefully inserted so that no air space was left in the system and the sac suspended in the bottle containing the outer fluid. The initial pressure in the manometer was due to the insertion of the manometer tube but this pressure rose or fell in a short time to the true osmotic pressure of the solution of haemoglobin. The osmometer and manometer could easily be removed from the bottle containing the outer fluid and held while changing the water in the bottle. Sufficient water was used completely to cover the collodion sac (about 100 cc.).

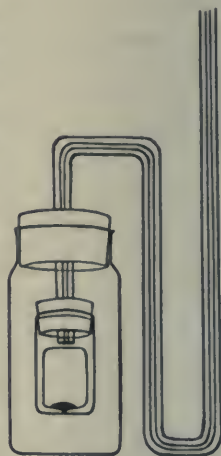


Fig. 1.
Type B Osmometer

A comparison was made of preparations of haemoglobin obtained by the alcohol method and the Dudley and Evans method. Solutions of approximately the same strength (9 %) were placed in Type A osmometers and treated in the same manner in each case. The alcohol preparation had been crystallised four times and the D. and E. preparation three times. The following table will show that there is no appreciable difference in the osmotic pressure of the two solutions, either against distilled water or after treatment with acids.

Time of reading osmometers		Outer fluid	Osmotic pressure in mm. Hg	
			Alcohol preparation	D. and E. preparation
18. xi. 21	5 p.m.	Distilled water	39	52
19. xi. 21	1.40 p.m.	"	40	44
21. xi. 21	3.15 p.m.	Water + CO ₂ ($p_H = 4.0$)	64	68
22. xi. 21	10.35 a.m.	"	76	81.5
23. xi. 21	12.40 p.m.	"	93	100
Later in same experiment:				
12. xii. 21	10.45 a.m.	Distilled water	52	54
13. xii. 21	11 a.m.	Acetic acid ($p_H = 4.0$)	68	72
14. xii. 21	4.20 p.m.	" "	74	81
15. xii. 21	11.15 a.m.	Distilled water	70	76
16. xii. 21	10 a.m.	" "	68	66

A comparison of oxyhaemoglobin and reduced haemoglobin was made. In this case the osmometers of Type B were used. The reduced haemoglobin was prepared from the same 2 % stock solution as the oxyhaemoglobin, by exposing a small amount of liquid to a vacuum for three and a half hours. In each case the pressure rose to practically the same extent (51–53 mm. Hg) with the use of CO₂. This was to have been expected, as the use of a saturated solution of CO₂ as an outer fluid probably changed the oxyhaemoglobin to the reduced form.

EXPERIMENTAL.

Below is given a detailed description of a characteristic experiment showing the influence of CO₂ and acetic acid on haemoglobin. A weak solution of haemoglobin was prepared by adding water to crystals of oxyhaemoglobin (four times crystallised) and centrifuging to get a clear solution free from crystals. By determining the percentage of solid and ash in 10 cc., it was found to be a 1 % solution of haemoglobin. Without delay, six osmometers of Type B were filled with the same solution. Distilled water of p_H 6.8 was used in all the osmometers as the outer fluid. On the second day of the experiment, and thereafter, oxygen was bubbled through the distilled water before using the osmometers, to keep the oxyhaemoglobin saturated with oxygen and to remove the CO₂ dissolved in the water from the air. When equilibrium had been obtained (the outer water having been changed frequently for two days), all six osmometers gave practically the same osmotic pressure (7 to 11 mm. Hg). This agrees quite well with the calculated osmotic pressure (10.9 mm.) of a 1 % haemoglobin solution at 20°, using 16,669 as the minimum calculated molecular weight of haemoglobin [Gamgee, 1898; Hüfner and Gansser, 1907;

Barcroft and Hill, 1910]. The experiments were divided into two groups. Three osmometers Nos. 22, 23 and 24 were used for group I, and the other three, Nos. 25, 26 and 27, for group II. One osmometer from each group (Nos. 22 and 25) was kept in an ice-box at 4° throughout almost the entire experiment. The data for these experiments are given graphically in Fig. 2 and Fig. 3.

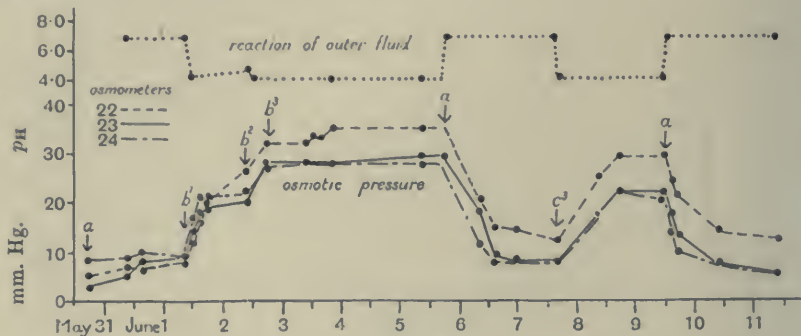


Fig. 2.

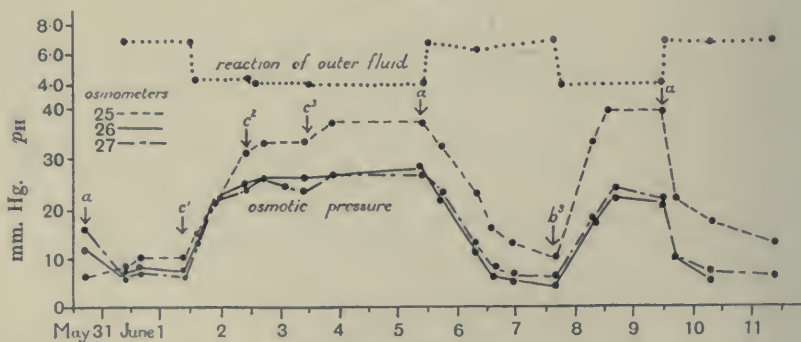


Fig. 3.

Figs. 2 and 3. Effect of CO_2 and acetic acid on the osmotic pressure of haemoglobin. Outer fluids:—*a* = distilled water ($p_{\text{H}}=6.8$); *b*¹ = water 1/10 saturated with CO_2 ($p_{\text{H}}=4.2$); *b*² = water half saturated with CO_2 ($p_{\text{H}}=4.0$); *b*³ = water fully saturated with CO_2 ($p_{\text{H}}=3.8$); *c*¹ = acetic acid ($p_{\text{H}}=4.2$); *c*² = acetic acid ($p_{\text{H}}=4.0$); *c*³ = acetic acid ($p_{\text{H}}=3.8$). The arrows indicate the first introduction of a particular outer fluid which was changed several times a day until a new outer fluid was used.

In the osmometers of group I, water one-tenth saturated with CO_2 ($p_{\text{H}}=4.2$) was used as outer fluid. This was made by adding nine parts of distilled water to one part of water saturated with CO_2 . Care was taken to minimise the loss of CO_2 but no special precautions were used. The osmotic pressure rose to 20 and 22 mm. Hg in the osmometers at room temperature and to 26 mm. Hg in the one in the ice chest. Half saturated CO_2 solutions ($p_{\text{H}}=4.0$) were used next, followed by fully saturated ($p_{\text{H}}=3.8$), each of which caused a further increase of pressure as may be seen in Fig. 2. This pressure remained constant until, two days later, distilled water was substituted, causing the pressure to fall to its former value. Next, acetic acid of the

same p_{II} (3.8) as the last carbonic acid solution was used. Although the molar strength of the acetic acid was only 0.0013, compared with 0.042 M of the CO_2 solution, the osmotic pressure rose to practically the same extent in each case. This seems to indicate that this rise in osmotic pressure is due not to the particular acid used, but to the hydrogen-ion concentration of the solution. One experiment with 0.00037 M lactic acid ($p_{II} = 3.8$) gave similar results.

The order of the experiments was reversed with the second group of osmometers (see Fig. 3). After determining the osmotic pressure of the solution of haemoglobin against water, the p_{II} of the outer fluid was decreased as in the experiments in group I, using acetic acid instead of CO_2 . The p_{II} of the solution was changed first to 4.2, then 4.0 and 3.8, with concentrations of acetic acid varying from about 0.0005 N to 0.0013 N . After maintaining this pressure for several days, distilled water was used to remove the acid and, finally, water saturated with CO_2 was put into the outer chamber of the osmometer. It can be seen from the figures that a rise in osmotic pressure occurs, and the rise is practically the same whether one acid or the other is used first as an outer fluid. In osmometer No. 25 the osmotic pressure, when CO_2 was used, was four times the value against water. The systems were considered to be in equilibrium when, after repeated changes of the outer fluid, the hydrogen-ion concentration became constant. The p_{II} of the outer fluid was determined colorimetrically, using bromophenol blue, bromocresol purple, and phenol red as indicators.

Results similar to those described above were obtained with a number of solutions of haemoglobin of different strengths using both types of osmometer. Haemoglobin prepared by both methods was used. With the stronger solutions the results are similar to those obtained with the 1 % solutions, but there was never proportionally as great a rise in the osmotic pressure produced by acids.

It is important to know whether or not the haemoglobin was decomposed or changed in any way during the experiments. There was no sign of decomposition other than the formation of a small amount of precipitated haemoglobin after acids were used. In the osmometers kept in the cold (Nos. 22 and 25) this amount was very slight. It was evidently too small appreciably to affect the osmotic pressure, for, whenever the solutions in these osmometers were dialysed against water, after the use of acids, the osmotic pressures returned to their former values. In the osmometers at room temperature, on the other hand, after repeated use of acids as the outer fluid, there was sufficient precipitation to account for a progressive reduction in the values against water, as, for example, in osmometer No. 24 from 9—8 to 5 mm. Hg.

To see whether or not oxyhaemoglobin changes on merely standing, two osmometers were filled with the same 1 % solution used in the experiments described in detail above, and were left standing, occasionally changing the distilled water which was used as the outer fluid. The solution at the end of several weeks was apparently unchanged. There was no precipitate and the solution gave the spectrum of oxyhaemoglobin.

Haemoglobin solutions, saturated with CO_2 , are known to become reduced [Preyer, 1868]. But Heidelberger [1922] states that an oxyhaemoglobin solution will not become reduced when a mixture of 20 % oxygen and 80 % carbon dioxide is bubbled through the solution. In the experiments when water 1/10 saturated with CO_2 was used, there was probably enough oxygen present (18–19 %) to keep the haemoglobin completely oxygenated. When, however, water saturated with CO_2 was used, the oxyhaemoglobin was doubtless changed almost completely to reduced haemoglobin.

When air is again admitted to a solution of haemoglobin, through which CO_2 has been bubbled, methaemoglobin is formed [Preyer, 1868]. Oxyhaemoglobin treated with acetic acid [Menzies, 1895] is also changed to methaemoglobin. Hence it is not surprising that at the end of the experiments, after repeated use of CO_2 and acetic acid, the solution in the osmometers was found to give the spectrum of methaemoglobin.

Since a rise in osmotic pressure occurred in all the experiments when acids were used, whether or not oxyhaemoglobin, reduced haemoglobin, or methaemoglobin were present, evidently the effects of the acids are similar with each compound.

DISCUSSION.

The increase in the osmotic pressure of a haemoglobin solution, when treated with CO_2 or with traces of acetic acid must, obviously, be due to the presence of a greater number of osmotically active particles than before. This might possibly be brought about in one of a number of different ways.

1. As the membrane is permeable to the molecules and ions of H_2CO_3 and acetic acid¹, they cannot exist in greater concentration inside the osmometer than outside it. Hence the acid used cannot be responsible for the rise in osmotic pressure.

2. If the haemoglobin had been aggregated in water and the acid had caused de-aggregation, there would have been an increase in osmotically active particles. Both assumptions are, however, contradicted by certain evidence. The observed osmotic pressure against water was approximately the same as the osmotic pressure calculated on the assumption that the haemoglobin existed as single molecules. During the course of the osmotic pressure experiments with acids, the gradual formation of a small amount of precipitate was observed in the osmometers, showing that a certain amount of aggregation had gone far enough to cause gross precipitation, and this would, of course, tend to cause a fall in osmotic pressure.

The work of Barcroft gives additional evidence on both these points. Barcroft [1914, Appendix II, pp. 314–315], gives the values for n in Hill's [1913] formula,

$$y = \frac{100kx^n}{1 + kx^n}$$

for the dissociation curves of dialysed haemoglobin at 40° , equilibrated at

¹ For the sake of brevity, since the results were the same with CO_2 and acetic acid of the same p_{H} , the discussion will hereafter be confined to the experiments with CO_2 .

various CO_2 tensions. At 0 mm. CO_2 tension $n = 1$, at 8 mm. $n = 1.78$, at 33 mm. $n = 2.5$, and at 67 mm. $n = 2.7$. Barcroft [1913, p. 481] gives "the average number of molecules of haemoglobin in each aggregate" as the definition of " n ." According to these calculations, therefore, haemoglobin exists as single molecules in pure watery solution and is aggregated by CO_2 ¹.

Although, in the experiments described in detail in this paper, a weak solution of haemoglobin (1 %), dialysed against distilled water gave an osmotic pressure practically identical with the calculated value, stronger solutions (2-14 %) frequently gave lower pressures than those calculated for the particular concentration of solution used, indicating that some aggregation had taken place. Reid found values only one-third the calculated for 3-6 % haemoglobin solutions, and concludes [1905, p. 18] "that the molecular weight in solution in water would appear to be a multiple of the minimum calculated from analysis." Aggregation seems a more probable explanation.

In this connection it is interesting to note that Lillie found a fourfold increase in the osmotic pressure of a gelatin solution, when sufficient HCl was added to the inner and outer chambers of the osmometer, to make $M/770$ HCl solutions. He says [1908, p. 140] "The increased pressure may safely be regarded as due to a simple change in aggregation state." He does not suggest ionisation as a possible cause. Either de-aggregation or ionisation would give an increased osmotic pressure. The osmotic pressure of his gelatin solutions returned to their former values if the acid was removed by dialysis against water. There was no increase, and sometimes even a decrease, when egg albumin was treated similarly.

3. Decomposition of the haemoglobin into smaller molecules, which were still too large to pass through the collodion sac, would give an increased number of osmotically active particles. The fact that the osmotic pressure can be brought back practically to its former value (see especially No. 25), by substituting water for the acid, is evidence that decomposition plays little or no part in the rise of osmotic pressure. Haemoglobin decomposed into haematin and globin, for instance, would not re-unite to form haemoglobin by merely substituting water for acid in the outer chamber of the osmometer.

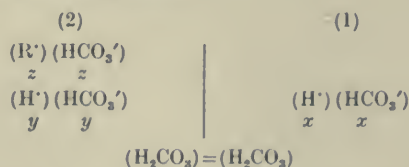
4. Martin [1896] has shown that the osmotic pressure of haemoglobin varies with the absolute temperature. Roaf [1912] has shown that the osmotic pressure of haemolysed pig's corpuscles rises from 6.9 mm. (maximum pressure per 1 % of organic matter at 16°) to 10.5 mm. Hg at 37°. Thus, even with wide variations in temperature the variation in osmotic pressure is not great. Furthermore, in the experiments reported in this paper, the temperature seldom varied more than a few degrees, and in no case was there parallelism between a rise of temperature and a rise in osmotic pressure.

¹ *Now added Feb. 5th, 1923.* This aggregation should cause a lowering of osmotic pressure. The rise of osmotic pressure, which is observed in these experiments, must occur in spite of the aggregation caused by the CO_2 . The rise in osmotic pressure cannot, therefore, be as great as it would have been had there been no aggregation.

5. There still remains the possibility of the formation of ions by (a) ionisation of haemoglobin bicarbonate or (b) ionisation of the haemoglobin molecule into protein ions. These possibilities the author now proposes to discuss in some detail.

(a) Since haemoglobin is an amphoteric electrolyte it may be assumed that it can combine with acids to form salts. It has been shown that the osmotic pressure of haemoglobin against CO_2 may be nearly four times as high as against water. If haemoglobin bicarbonate is formed, the salt must have, under these circumstances, an average of three bicarbonate radicles to every haemoglobin radicle in order to furnish enough ions to bring about the increased pressure. A study of Donnan's theory of membrane equilibrium [1911] will show that there would be, under these circumstances, a counter osmotic pressure that must be taken into consideration. Donnan and Harris [1911] and Bayliss [1911] have shown that there is unequal distribution of an electrolyte, such as NaCl , between a solution of the sodium salt of Congo-red inside, and water outside, a membrane impermeable to the Congo-red. From thermodynamic considerations, Donnan [1911] showed that the product of the concentrations of the Na and Cl ions inside the membrane must equal the product of their concentrations outside the membrane. Since a part of the Na ions inside the membrane comes from the sodium salt of Congo-red, then the concentration of the ionised NaCl inside the membrane, will be less than that outside the membrane. This excess of NaCl outside gives rise to a counter osmotic pressure and therefore the osmotic pressure of the Congo-red salt will be less than the theoretical. The extent of the depression of the osmotic pressure depends on the relative concentrations of the colloid and electrolyte.

Applying this theory to our experiments, we have the following: where R = the cation of haemoglobin bicarbonate, (1) = outer fluid, (2) = inner fluid, and x , y and z are ion concentrations.

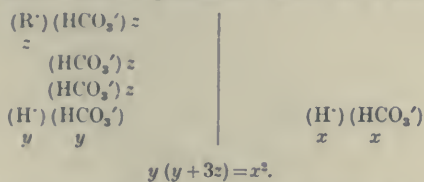


According to Donnan and Allmand [1914] the concentration of the undissociated electrolyte is the same on both sides of the membrane, and hence will not affect the osmotic pressure in any way. The ionic equilibria may be expressed:

$$y(y+z) = x^2.$$

Under these circumstances, instead of the osmotic pressure being dependent only upon the concentration $2z$, there is a counter osmotic pressure represented by $2x - 2y$. Furthermore, the H -ion concentration (y), inside the membrane, is less than the hydrogen ion concentration (x), outside the membrane. The following calculations, made on the basis of the foregoing, show

the extent of the counter osmotic pressure and the difference in p_H on the two sides of the membrane. The haemoglobin bicarbonate is this time written with three bicarbonate radicles for each haemoglobin radicle to account for the four-fold increase of osmotic pressure observed while using CO_2 .



When the p_H of the outer fluid is 4, $x = 0.0001 N$. A 1 % haemoglobin solution (molecular weight 16,000) is 0.0006 M . Assuming complete ionisation of the haemoglobin bicarbonate, $z = 0.0006 N$. Substituting in

$$\begin{aligned}
 y(y+3z) &= x^2, \\
 y(y+0.0018 N) &= 0.000,000,01 N, \\
 y^2 + 0.0018 N y &= 0.000,000,01 N,
 \end{aligned}$$

y^2 being infinitesimal as compared with the other values, may be disregarded. Hence

$$y = 0.000,006 N.$$

The counter osmotic pressure is dependent upon the excess of ionised H_2CO_3 outside the membrane over that inside the membrane

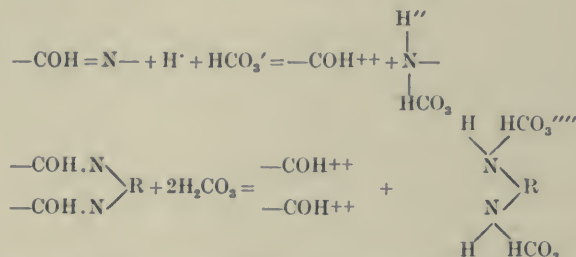
$$2x - 2y = 0.000,18 N.$$

The osmotic pressure of the haemoglobin bicarbonate, which is dependent upon the concentration $4z$ (0.0024 N), would, in this case, be reduced about 10 % due to the unequal distribution of the ionised H_2CO_3 . For example, if the osmotic pressure had been 10 mm. Hg against water, and a haemoglobin bicarbonate were formed (such as described above), the pressure instead of rising to 40 mm. Hg would rise to 36 mm. Hg. In these experiments, therefore, since the x concentrations are much less than the z concentrations, the counter osmotic pressure does not interfere much with the osmotic pressure determinations. With stronger solutions of haemoglobin the counter osmotic pressure would be proportionately less. A 10 % solution would show 99 % of the calculated osmotic pressure.

The effect of the Donnan equilibrium on the H-ion concentration of the inner fluid is more marked in the example given above than is the effect on the osmotic pressure. y , the H-ion concentration of the inner fluid, is 0.000,006 N ($p_H = 5.2$), while x , the H-ion concentration of the outer fluid is 0.0001 N ($p_H = 4.0$). With a 10 % solution of haemoglobin, in the form of haemoglobin bicarbonate, the inner fluid would have a p_H of 6.2. Since the isoelectric point of haemoglobin is about p_H 7.0 (Michaelis and Takahashi [1910] give 1.9×10^{-7} ($p_H = 6.75$) and Campbell and Poulton [1920] $p_H = 6.98$), undoubtedly a salt could be formed at a p_H of 5.2. A p_H of 6.2 might also be sufficiently acid for salt formation, although Bayliss [1921] has shown, for

dialysed blood serum, that there is a considerable lag in salt formation with acids, until the p_H is well on the acid side of the isoelectric point.

(b) Considerable evidence for the dissociation of proteins into two or more protein ions, due to the action of acids and alkalis, is given by Robertson. He says [1918, p. 20] "we may now regard it as an established fact that some elements in the protein molecule other than the terminal $-\text{NH}_2$ or $-\text{COOH}$ groups are responsible for the acid- and base-neutralising power which is possessed in such a marked degree by many proteins." Substituting H_2CO_3 for HCl in equations ii (p. 25) and vi (p. 26) we have:



In this case we would presumably have no Donnan effect, as the freely-diffusing dissociated carbonic acid has no ion in common with the haemoglobin, which has dissociated into protein ions. With the equal distribution of H and HCO_3 ions on both sides of the membrane there should be the same H -ion concentration on both sides. If the above theoretical considerations are sound, it should be possible to decide what kind of dissociation of haemoglobin has taken place by testing the p_H of the haemoglobin solutions in the osmometers, by means of the hydrogen electrode. If only protein ions are formed, the H -ion concentration of the inner fluid should be the same as the outer fluid. If haemoglobin bicarbonate is formed, and the Donnan equilibrium comes into play, the inner solution should be much less acid than the outer. The same relations should hold true for a haemoglobin acetate also. The author proposes to investigate these points shortly.

CONCLUSION.

It is shown that the osmotic pressure of solutions of purified haemoglobin is three or four times as great when dialysed against acetic acid, or water saturated with CO_2 (both about p_H 4), as against water alone. Reasons are given for believing that this is due to the formation of some sort of an ionising salt of haemoglobin, either one which ionises into several protein ions, or into protein and acetate or protein and bicarbonate ions.

This investigation was undertaken at the suggestion of Professor Sir William Bayliss, to whom the author wishes to express her thanks for encouragement and advice throughout the course of the work.

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IX. AMMONIA CONTENT OF THE BLOOD IN NEPHRITIS.

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(Received January 5th, 1923.)

IN October, 1921, Nash and Benedict [1921] published a paper in which they gave an historical review of previous work concerning the ammonia-content of blood, together with work which they themselves had done on this subject, using dogs under various conditions. They showed that low figures, ranging from 0.03 to 0.14 mg. per 100 cc., were obtained for the blood-ammonia in normal cases, and that in nephrectomised dogs the values were markedly reduced. They also found a higher percentage of ammonia in blood from the renal vein than elsewhere in the circulation. On these data they put forward the view that the kidneys are the site of ammonia-formation in the body, the small quantities present in the blood being regarded as an overflow from these organs into the systemic circulation.

In view of this hypothesis it was considered that a series of estimations of the blood-ammonia in cases of severe nephritis would be of interest. In selecting the cases, preference was shown for those with a marked nitrogen-retention, as it was thought that these would be the most likely to give abnormal figures. In the series quoted other details relating to the renal efficiency of the patients are given wherever available.

Method.

The Folin-Denis aeration method was employed, as described by Nash and Benedict. With the exception of Case 3, the time elapsing between the drawing of the blood and the beginning of aeration was not greater than 10 minutes. In the case mentioned the interval was five minutes longer, and the sample was placed on ice during this time. An electric suction pump was used for the aeration, and in all cases this step in the analysis lasted 15 minutes.

Controls.

Controls for the method, as employed, were carried out as follows:

(1) 5 cc. of a standard ammonium chloride solution, containing 0.014 mg. N (or 0.28 mg. N %) were used for an analysis in place of 5 cc. of blood. The resulting solution in the acid-holding tube, when Nesslerised, gave an exact match with 5 cc. of the untreated standard simultaneously Nesslerised.

(2) A sample of blood from a patient without known renal disease (Case 1, Table II) was analysed, and the ammonia estimated immediately. Without further delay 0.5 cc. of diluted ammonium chloride solution, containing

0.014 mg. N was added to the aerated blood, and a second aeration immediately performed, adding another 1.0 cc. of carbonate-oxalate solution. The resulting solution in the acid-holding tube gave an exact match with the same quantity of diluted standard in 5 cc. of ammonia-free water, simultaneously Nesslerised. This experiment was repeated on Case 11, Table II, adding 0.5 cc. containing 0.0047 mg. N, with the same result.

(3) Since it was suggested that this result might have been obtained as the result of incomplete removal of the blood-ammonia during the first aeration, the following investigation was made:

5 cc. of freshly drawn blood (Case 2, Table II) were aerated, and the blood-ammonia immediately estimated. With the least possible delay, a second aeration was performed on the same blood for another 15 minutes. The fluid in the acid-holding tube showed no trace of colour on Nesslerisation.

This experiment demonstrated not only the adequacy of the method as a means of analysis, but the purity of our reagents with respect to ammonia.

It should be added that, where the concentration of blood-ammonia was under 0.04 mg. N %, the values given are only approximate, since the depth of colour on Nesslerisation was too faint to admit of accurate estimation.

Table I gives figures for a series of 17 cases examined in the wards of the hospital. Of these Cases 1, 5, 12 and 17 died, but in only the last two mentioned was a post-mortem obtained. The diagnosis of chronic interstitial nephritis was confirmed, but the picture was complicated in Case 12 by the additional finding of infective endocarditis, which probably superimposed an acute infection on the already damaged kidneys.

These same four cases presented symptoms clinically recognised as uraemia, *i.e.* pronounced drowsiness with irregular muscular twitchings, uraemic smell and irregularities in respiration. In Case 12 this irregularity was Cheyne-Stokes in type. Case 1 showed a urea frost during 48 hours before death, a rare phenomenon in which urea crystallises on the surface of the skin giving it a rough, whitish appearance. Case 13 is of considerable interest, since the patient was not confined to bed, but actively engaged in domestic service and not apparently inconvenienced by her renal inefficiency. In other respects the table is self-explanatory. In estimating $p_{H_2CO_3}$ of blood and plasma bicarbonate the method of Van Slyke and Cullen was employed [1922]. Table II gives a series of 11 cases in which the blood-ammonia was estimated in cases with no evidence of renal disease. Cases 5, 6, 7 and 8 were normal people, the rest were patients suffering from the diseases indicated. The number of cases investigated in this second series is not sufficient to enable one to draw conclusions, but one is led to think that the ammonia-content of the blood in normal people is almost negligible, and that the blood of ill people contains amounts of ammonia which, though small, will admit of estimation. In this class the value obtained for the blood-ammonia seems to be independent of

Table I. *Values for blood-ammonia in cases with renal disease.*

Case	Diagnosis	Blood				Urine						Remarks
		Urea in NH_3 in mg. per cent.	HCO_3	pH	Volume 24 hours	pH	R-N	Titrateable acid of 0.1 N per cent.	Ammonia in cc. of 0.1 N in 1st 2 hours	P.S.P.		
1	Chronic nephritis	480	0.044	7.35	1620	—	1.96	19.6	10	—	4 days later	
		600	0.044	7.35	1920	4.8	2.1	18.8	9	0	9 days later. Died	
		600	0.088	7.4	1620	—	2.8	20	7	—		
2	Chronic interstitial nephritis	66	0.068	7.55	—	6.8	—	—	—	—	15 mins. delay before aeration	
3	" "	41	0.044	—	—	—	—	—	35	—		
4	Toxaemia of pregnancy	33	0.044	—	—	—	—	—	—	28		
5	Chronic interstitial nephritis	564	0.05	35	7.4	Alkaline. Bacterial action	3.4	54.8	16	15.5	Died	
6	Chronic parenchymatous nephritis	120	0.044	61	7.4	—	1.0	8	8	21.7		
7	Pyonephrosis	41	0.066	59	7.4	—	4.9	1.5	25	16	32	
8	Subacute	55	0.04	—	—	4.8	1.3	25	19	—		
9	Chronic interstitial nephritis	92	0.045	—	—	Alkaline	—	—	—	—		
10	Acute	120	0.066	67	—	—	3.1	34	11	11.5		
11	Chronic interstitial nephritis	63	0.044	—	610	Alkaline	—	—	—	Trace		
12	" "	171	0.088	43	7.6	—	—	—	—	—	5 days later. Died	
		240	0.044	54	7.4	—	—	—	—	—	Urine doubtful. Acid NH_3 on previous occasions	
13	Congenital bilateral cystic kidneys	141	Nil	33	7.2	—	0.16	2	12	Trace		
14	Subacute	67	0.088	—	1300	—	4.9	39	8	16.8		
15	Chronic nephritis	171	0.09	62	7.4	—	2.6	16	6	7.4		
16	Infective endocarditis with renal damage	63	0.02	46	7.6	5.6	0.34	14	41	39	7 days later	
		52	0.03	42	7.5	—	—	—	—	—		
17	Chronic interstitial nephritis	343	Nil	13	7.2	Alkaline ? bacterial action	—	—	—	—	4 days later. Bicarbonate given in interval	
		400	0.04	74	7.55	—	—	—	—	0	10 days later. Died	
		600	0.02	41	7.35	—	—	—	—	—		

the nature of the illness, and the range of figures for the non-renal cases coincides with the range obtained for renal cases. More work along this line would be useful. It is possible that such factors as toxæmia might be found to play a part.

Table II. *Values for blood-ammonia in cases without renal disease.*

Case	Diagnosis	Blood-NH ₃ in mg. N %	Remarks
1	Addison's disease	0.05	
2	Rheumatic fever	0.088	
3	Catarrhal jaundice	0.02	
4	Diabetes mellitus	0.02	No ketosis present
5	Normal	Very faint trace	} Blood drawn in laboratory and transferred to pump immediately
6	"	Nil	
7	"	Faint trace	
	[repeated]	" "	Stood 10 mins. before aeration
8	Normal	" "	
9	Carcinoma of stomach	" "	
10	? Duodenal ulcer	" "	
11	Hepatic enlargement	0.05	

DISCUSSION.

If we adopt the view that the ammonia present in the urine has been preformed in the blood and simply excreted by the kidneys, it seems legitimate to expect that cases with marked urea-retention would also show ammonia-retention to a comparable degree, particularly if the formation of urea from ammonia is a reversible reaction. If, however, Nash and Benedict [1921] are correct in their hypothesis that the kidneys are the site of ammonia-formation, we might reasonably suppose that there would be no ammonia-retention corresponding to high values of the blood urea, since ammonia-formation would be suppressed in common with other renal functions.

A consideration of Table I at once shows that no ammonia-retention has been found in any of the cases. The values obtained vary from 0.09 mg. N % to zero and are of the same order as those of the cases in Table II.

Next, if the blood-ammonia is not renal in origin, but is derived from other organs it might be expected, on the filtration hypothesis, that the ratio between urea and ammonia in the urine would bear a close relation to the same ratio in the blood.

Taking as normal figures:

Urea in urine	2000 mg. %.
Ammonia	33 mg. %.
Urea in blood	25 mg. %.

Then ammonia in blood = $\frac{33 \times 25}{2000} = 0.4$ mg. %.

This figure is over four times as great as the maximum value observed.

Also, if ammonia is preformed in the blood, we ought to find a definite relation between the values for blood-ammonia and urinary ammonia. In case 16, the excretion of ammonia in a 24 hourly specimen was equivalent

to 57.4 mg. N %, whereas the blood-ammonia was only 0.02 mg. N %. Supposing her weight to be 50 kilos, and the volume of her blood 4,250 cc. (85 cc. per kilo) the total ammonia-content of her blood would be 0.85 mg. N. The whole volume of her blood must therefore circulate through the kidneys completely 384 times per 24 hours, and the kidneys must be 100 % efficient in removing all ammonia from the blood, in order to make up the figure for the urine ammonia. Or, in other words, the total blood flow through the kidneys must be 1632 litres per 24 hours. This is a possible rate of flow, according to Starling [1915], but it is highly improbable that a kidney with such impaired functions as are shown in this case could rise to a 100 % efficiency with respect to ammonia excretion.

Further evidence may be adduced from the consideration of the ammonia-content of urine and blood in acidosis. A large percentage of the total acidity of the urine is represented by ammonia as ammonium salts. This amounts, in normal persons, to about 30 mg. %. The ammonia in the blood, at a generous estimate, is present to the extent of 0.1 mg. %. But if ammonia is used as a neutralising agent for non-volatile acids in the blood it might be expected that, where increased acid is circulating, there would be a proportionate increase in the blood ammonia. Thus case 17 shows a marked acidosis with a decrease in the plasma bicarbonate (taking the normal as 60 vols. %) equal to 0.02 gram-equivalents per litre. If only a quarter of this were neutralised by ammonia (25 % being a low value for the percentage of acid neutralised by ammonia in urine) the figure for the blood-ammonia nitrogen would equal 0.005 gram-equivalents per litre = 0.005×14 g. N per litre = 0.007 g. N %. This is 70 times in excess of the maximum value obtained in any case in the series. Similarly, arguing on the same lines, the figure for Case 13 would be 0.004 g. ammonia N %, and in Case 5 it would be 0.0049 g. ammonia N %. Both of these are approximately 40 times in excess of the values found. It is perhaps noteworthy, in this connection, that Cases 17 and 13 are the only cases in Table I in which no trace of blood-ammonia could be found.

SUMMARY.

1. Twenty-three estimations of the blood-ammonia for a series of 17 cases are recorded, in patients showing advanced renal disease. In all cases the values reported are under 0.1 mg. % and are comparable to the values recorded for eleven cases with no known renal disease.
2. Controls for the technique employed are described.
3. It is argued that the evidence available lends support to Nash and Benedict's hypothesis that the kidneys are the site of ammonia formation in the body, and that the traces found in the systemic circulation represent an overflow from these organs.

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X. TETHELIN:—A GROWTH-CONTROLLING SUBSTANCE OBTAINABLE FROM THE ANTERIOR LOBE OF THE PITUITARY BODY.

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(Received January 5th, 1923.)

IN a recent communication, Drummond and Cannan [1922] have published an account of certain experiments and critical considerations which, in their estimation, invalidate the conclusions drawn by the author concerning the effects of pituitary (anterior lobe) substance upon the growth of animals [1916, 2, 3; Robertson and Ray, 1919] and the presence of a growth-controlling agent in the precipitate produced by ether from an alcoholic extract of the anterior lobe tissue (Tethelin).

The paper of Drummond and Cannan consists of two parts, the one dealing with the question of the composition of tethelin, and the other describing their failure to influence the growth of mice by the administration of pituitary (anterior lobe) tissue. These sections of their paper are presented as if they were logically related and mutually confirmatory, but in fact, save by the common bond of indiscriminate criticism, they are in no way connected. I propose to deal with the two parts separately in the order in which they are presented by Drummond and Cannan.

Isolation of tethelin.

In my original article describing the isolation of tethelin from the alcoholic extract of pituitary (anterior lobe) tissue [1916, 4] I was at pains to point out that the substance is exceedingly susceptible to oxidation. Drummond and Cannan declare that they "have made several attempts to prepare this substance and to obtain a white precipitate such as Robertson described by following the details of his method." This phraseology appears to be meant to create the impression that they failed. The obvious course for Drummond and Cannan to pursue would have been to procure the substance from the manufacturer¹, in which case they would have ascertained that others, besides myself, have been able to prepare this product without pronounced dis-

¹ H. K. Mulford and Co., Philadelphia.

coloration¹. Instead of this, they introduced an "improvement" into my method and employed an atmosphere of carbon dioxide throughout its preparation. Now, as a matter of course, this was the first method which occurred to me to protect tethelin from oxidation, and it was abandoned because I found that carbon dioxide itself caused softening and darkening of the product. A possible reason for this became apparent later, when Schmidt and May [1917] found that heating of tethelin, even in faintly acid solutions, induces decomposition which may be rendered manifest by physiological tests².

From the material which they prepared in this manner Drummond and Cannan now endeavoured to separate several fractions in order to demonstrate that it was a mixture of substances. After hydrolysis by alkalis and by acids I also have separated various fractions from the hydrolysate, and these experiments are described in detail in my paper. After procuring three fractions which presented differing characteristics they abandoned further attempts, owing to their "conviction that the matter was not worthy of further consideration." In agreement with this conviction we may terminate our discussion of this portion of Drummond and Cannan's contribution.

Feeding experiments.

The question of the chemical unity or composition of tethelin is, of course, not in the least involved in the separate question whether or not it contains the whole or a portion of those substances in the pituitary gland which are capable of affecting the growth of tissues. This latter question has been approached in my experiments by the direct administration of tethelin to normal growing animals and to animals inoculated with carcinoma. Drummond and Cannan approach it by the administration of pituitary (anterior lobe) tissue to young mice. In their expressed belief their results invalidate conclusions which have been based by numerous observers besides myself upon experiments extending over many years upon a variety of animal types. They state in the first place that administration of the tissue of the anterior lobe of the pituitary body to animals is devoid of effect upon their growth, in contradiction to the previous findings of Schafer [1912], Cushing [1909], Aldrich [1912], Wulzen [1914], Maxwell [1916], Goetsch [1916], Marinus [1919], Pearl [1916], Uhlenhuth [1921, 1922], and Robertson and Ray [1916, 2, 1919, 1920], all of whom have reported more or less decided effects upon growth as the result of such administration. One would expect to find an opinion, advanced in opposition to such united testimony, supported by a wealth of experimental evidence and an exhaustive analysis of technical detail, but, on

¹ Under laboratory conditions discoloration may be totally avoided, but to attain this the alcohol and ether used must be neutral and anhydrous, the salts employed must be strictly neutral (we manufactured our own calcium sulphate for this purpose) and a heated and evacuated desiccator must be employed [Robertson and Schmidt, 1916].

² Doubtless an atmosphere of dry nitrogen would overcome these difficulties, but we have not found it necessary to resort to this.

the contrary, the evidence presented, in so far as any quantitative evidence is advanced at all, is derived from data furnished by the growth of *ten* animals of each sex during a period of administration which extended over nine weeks¹.

It is urged by Drummond and Cannan that the testimony of former observers is conflicting, in that some report acceleration of total growth as a result of administering pituitary tissue, while others report retardation. The testimony is not conflicting, however, if we view it in the light of the experiments of Schafer, Wulzen, Maxwell, and Robertson and Ray, which have shown that the retardation occurs at one age (and possibly affects one kind of tissue), while the acceleration occurs at another. In seeking to ascertain a reason for Drummond and Cannan's failure to confirm the results of their predecessors one is struck by the fact that they employed *dried* anterior lobe tissue in their feeding experiments, while others have employed *fresh* tissue. Having regard to the susceptibility of tethelin to oxidation, upon which Drummond and Cannan have rightly laid so much emphasis, this procedure was hardly calculated to display its effects.

In the drying of pituitary tissue prior to its extraction with alcohol during the preparation of tethelin, the tissue is mixed with a large bulk of anhydrous calcium and sodium sulphates, whereby the period of drying is abbreviated and the tissue is covered and protected to a considerable extent from the access of oxygen. This procedure is naturally impossible when the tissue is to be administered to animals. When the unprotected tissue is dried with free access to air intense discoloration occurs and it may be conjectured that the greater part of the tethelin is destroyed.

Notwithstanding the inadequate character of their own technique, Drummond and Cannan have not hesitated to make indefensible assertions concerning the work which their experiments are designed to invalidate. In view of the controversial methods which they adopt it is curious to find that they accuse the author of "biased interpretation," for the following reasons.

In my first experiments upon the growth of mice a batch of 72 animals (36 males and 36 females) were taken from the breeding stock without selection² and set aside to serve as normal controls. Fresh batches of animals were taken from the same stock, as rapidly as the available numbers permitted, to serve as the subjects of experimental additions to the diet. These constituted, of course, so many separate experiments for which the one group of animals served as controls.

Now the variability of mice at four or five weeks of age is over 20 %³, which means that one animal in three may be expected to deviate by over 20 % from the average. This being the case, batches of 36 or 24 animals of

¹ The mothers of these animals were fed with pituitary tissue until the young were weaned (at three weeks). But the investigations of Robertson and Delprat [1917] have shown that administration of tethelin to the mother is without effect upon the growth of suckling young.

² Excepting that involved in the rejection of animals which were obviously sickly.

³ Computed as the ratio of the standard deviation to the average weight.

the same sex, chosen without selection at this age, could not possibly be expected to display equal average weights. To attain such an end would necessitate either selection of individuals chosen to display equal averages, a procedure of very questionable theoretical validity, or else the employment of impossibly huge numbers. Each experimental group, therefore, deviated initially in some degree from the normal group and these deviations were sometimes in a negative sense and sometimes in a positive¹.

This being the case, Drummond and Cannan proceed as follows: "For example it is significant that, in his experiments the effect of feeding egg-lecithin to batches of normal mice chosen at random at the age of four weeks showed a difference of 1.5 g. in mean weight, *i.e.* 13 % of body weight—a difference of the same order as the defection of weight of tethelin-fed mice at the maximum divergence of this group from the normal. Yet this, in the former case, is explained away as being due merely to the high variability at that age, whilst in the latter case it is reported as being a marked defection due to the effect of tethelin."

The maximum divergence to which Drummond and Cannan refer occurred at 15 weeks of age, when the variability had fallen to 9 %. The extreme variates had approached one another as they always do during growth [Robertson, 1916, 1; Thompson, 1917] and the animals by now represented a much more uniform type. They differed from the normals by an amount equal to no less than seven times the probable error of the difference computed from the variabilities of the normal and tethelin-fed groups. Yet, according to Drummond and Cannan, this means no more than a deviation of three times the probable error, observed in a different group of animals! To anybody acquainted with the elements of statistical analysis, however, a deviation of seven times the probable error is 19,000 times as significant as a deviation of three times the probable error, since in the first case there is but one chance in 420,000 that the observed deviation could have been accidental, while in the second case there is one chance in 22.

However, let us accept Drummond and Cannan's strange conclusion and suppose that the initial differences of highly variable young are continued in the same proportion in slightly variable animals of more advanced age. It then becomes of crucial importance to know what was the initial deviation of the *tethelin-fed* group from the normals. By a strange oversight Drummond and Cannan omit to mention this. The initial deviation of this experimental group (males) from the average of the normals, as stated in my tables, was + 0.60 g. (1.3 times the probable error of the difference), or $4\frac{1}{2}$ % of the mean weight at that age *in the opposite direction to the maximum divergence from the normal which was subsequently displayed*. How are we to interpret this? On

¹ The consequences of the extreme deviation noted in the lecithin-fed class, to which Drummond and Cannan refer, are carefully considered in our communication of 1919 under the headings "Effects of Egg Lecithin" and "Possible errors of sampling" [Robertson and Ray, 1919].

Drummond and Cannan's own showing the initial divergence of the tethelin-fed group from the normal actually emphasises the significance of the subsequent outcome, and yet they forbear from mentioning it.

The objection is urged that the ages of the mice employed were not identical, since some mice were three days in advance of or behind the others. This arises from my procedure in taking, for example, mice of from 25 to 31 days of age inclusive as having been weighed on the 28th day. This bracketing is employed throughout for obvious technical reasons. The maximum divergence of age is three days, an important item without doubt at three or four weeks of age. But will Drummond and Cannan seriously maintain that it is important at 15 weeks of age?

It is stated by Drummond and Cannan that many other examples of "biased interpretation" occur throughout my papers, but one further example will suffice. "In support of his contention that mice fed upon pituitary are, size for size, heavier than those normally fed, Robertson reproduces a photograph of two mice of the same age and linear dimensions, one a normal mouse weighing 30 g., the other a pituitary-fed mouse weighing 37 g. When it is pointed out that the weight of the normal was about the mean for his batch, whereas the other weighed 27 % more than the mean for his batch—i.e. was an extravagantly abnormal member—the fallacy of the argument is apparent."

How shall I satisfy these critics? My contention was that the condition of mice fed for a prolonged period upon pituitary tissue resembled that of acromegaly in that size-for-size, their weights were in excess of normal, while weight-for-weight they were smaller than normal. My photograph illustrated this difference of "build." What alternative photograph should have been taken? If I had taken a pituitary-fed mouse of average weight and displayed it beside a normal mouse of the same weight, then this would necessarily have been an "extravagantly abnormal member" of the control group. If I had displayed two mice resembling each other neither in weight nor size, the comparison would have been meaningless. If I had been able to exhibit mice from each group resembling one another both in size and in weight, the effect to which I drew attention would not have existed. It is clear that no means of comparison could have been so devised as to meet with the approval of Drummond and Cannan, but that by their criticism they have unintentionally contributed eloquent testimony to the alteration of bodily proportion which was induced in my mice by pituitary feeding.

CONCLUSIONS.

1. The experiments of Drummond and Cannan upon the composition and properties of tethelin are, in part, repetition of experiments previously performed by the author and, in part, vitiated by a faulty method of preparation.

2. The experiments of Drummond and Cannan upon the administration of anterior lobe tissue to mice afford no evidence either for or against the view that tethelin contains a growth-controlling constituent of the pituitary body.

3. The statistical method of comparing the growth of normal animals with that of animals in receipt of dietary additions must of necessity be superior to the haphazard method of comparing individual growth-curves or curves constructed from the growth of a few animals. It is, in fact, the only method properly applicable to this type of problem. The criticisms directed by Drummond and Cannan against the author's employment of this method are based in part upon misstatements of fact and in part upon misapprehension of the method.

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XI. A NOTE ON THE HYDROLYSIS OF PECTIN.

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IN a communication to this Journal [1921] the present author showed that when pectin was treated with cold, aqueous alkalis or with the enzyme, pectase, pectic acid was formed, together with methyl alcohol and acetone. It was stated, moreover, that "the proportion of acetone to methyl alcohol produced appeared to be about one part of the former to two of the latter," and, furthermore, that "it appears likely, therefore that pectin is the dimethyl-*iso*-propenyl ester of pectic acid." This conclusion was not based on any indirect method of estimation of either the methyl alcohol or the acetone, which cannot easily be determined accurately in the presence of one another, but was a considered conclusion based on the actual amounts of these substances obtained after their separation by a prolonged and careful fractional distillation and subsequent treatment of the "acetone fraction" with calcium chloride. The amount of material dealt with was large, and more than 3 g. of practically pure acetone were obtained. The author sees no reason to modify his original, somewhat guarded statement.

In view, however, of several personal communications from an authoritative American source the subject appears to require a little further explanation.

The author's statement regarding the formation of acetone on cold¹ hydrolysis was at first denied, but has been subsequently confirmed, here and in America, but it must be pointed out that too prolonged treatment of the pectin with water at a high temperature, especially in the presence of acid, results in a loss of both methyl alcohol and acetone. It has now been stated, however, that whether the percentage of methyl alcohol yielded by pectin is calculated from the amount of alkali required to hydrolyse the latter, or estimated by Zeisel's method, practically identical results are obtained. From this it is concluded that there is hardly room for an *iso*-propenyl group in the molecule. It will at once be seen, however, that the amount of alkali required to eliminate an *iso*-propenyl group is the same as that required for a methyl group. Moreover, when employing Zeisel's method, acetone (or an *iso*-propenyl group) is converted into *iso*-propyl iodide, which readily volatilises and yields the same amount of silver iodide as would a methyl group.

This behaviour of acetone does not seem to be generally realised, and it is considered advisable to draw attention to it as several previous workers have employed Zeisel's method for estimating the methyl alcohol yielded by pectin.

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¹ Acetone is yielded by a great variety of products on treatment with hot alkali.

XII. UREASE. PART I. THE CHEMICAL CHANGES INVOLVED IN THE ZYMOLYSIS OF UREA.

By WILLIAM ROBERT FEARON.

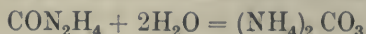
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UREA in aqueous solution is readily converted into ammonia and carbon dioxide by a definite class of ferments. The chemistry of this change attracted attention as far back as the end of the eighteenth century, when Fourcroy and Vauquelin (1798) proved that when urine undergoes alkaline fermentation on exposure to the air the ammonia formed is produced from the urea originally present in the urine.

Pasteur [1861] showed that a living organism was responsible for the alkaline fermentation of urine. The organism was isolated three years later by van Tieghem [1864], and was given the name *Micrococcus ureae*. Musculus [1874, 1876] obtained an enzyme from putrid urine, which he found capable of decomposing urea in aqueous solutions. Lea [1885] cultivated the *Micrococcus ureae* in quantity, and confirmed the results of Musculus.

Over 30 different micro-organisms capable of decomposing urea were recognised and described by Miquel [1890], who first suggested the name *urease* for the enzyme common to all these ureaclastic organisms. Since the discovery by Takeuchi [1909] of soy-bean urease, a plentiful supply of the enzyme has been assured, and much attention has been devoted to the study of the decomposition of urea by urease during the intervening years. Results of great practical value in connection with the estimation of urea have been obtained, but the actual chemical mechanism of the change does not seem to have been investigated very closely since Dumas [1830] suggested the equation



to represent the direct hydrolysis of urea in aqueous solution.

The zymolysis of urea has very generally been taken without question to be a simple direct hydration of the urea molecule, either free in solution or combined with the enzyme.

Armstrong and Horton [1912] considered the possibility of urea being in solution in the hypothetical hydrated form of "carbamide," $\text{C}(\text{OH})_2(\text{NH}_2)_2$, and producing on hydrolysis ortho-carbonic acid and ammonia.

Yamasaki [1920], from the reaction rates and temperature coefficients of the zymolysis, maintains that the decomposition of urea takes place in two definite stages, with the formation of ammonium carbamate as the inter-

mediate product. However, neither of the suggestions affects the general assumption that urea is broken down in solution by a direct hydration process.

If this be so, it seems rather remarkable that the attempts to obtain urea by a reverse synthesis from ammonium carbonate and carbamate, have been so unconvincing, more especially when it is considered that urea is a much more stable compound than either ammonium carbonate or carbamate.

Barendrecht [1919, 1921] has put forward a "radiation" theory of enzyme action. He asserts that when the urease "radiation" strikes a molecule of urea it is absorbed, and as a result the urea is hydrolysed. No explanation is offered as to how this hydrolysis takes place. In support of the "radiation" theory, Barendrecht claims to have observed a reverse zymolysis in solutions where the urease is "decaying," and to have obtained a synthesis of urea from ammonium carbonate solutions and urease "decaying" through the combined influences of alkalinity, time, and temperature.

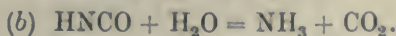
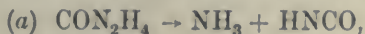
The validity of Barendrecht's claim is questioned by Mattaar [1920], who asserts that small quantities of urea when added to solutions of ammonium carbonate are destroyed by urease under conditions comparable with Barendrecht's alleged synthesis.

When it is realised that such a synthesis of urea is based on an entirely erroneous conception of the relationship between urea and ammonium carbonate, it will be seen that its occurrence is open to doubt.

The decomposition of urea in aqueous solution.

It was first proved by Fawsitt [1902] that the hydrolysis of urea by acids, alkalies, or water alone at the temperature of 100° does not proceed along the lines of the familiar equation suggested by Dumas in 1830. An intermediate cyanate stage was demonstrated, and Fawsitt concluded that urea in solution is first converted by an intramolecular change into ammonium cyanate, and that the ammonium cyanate is hydrolysed to ammonium carbonate. The influence of acids, alkalies, and alcohol on the change were also investigated [Burrows and Fawsitt, 1914].

E. A. Werner, in the course of his extensive work on the constitution of urea, has discussed these experiments in detail [1918], and has shown that the so-called "hydrolysis" of urea under all conditions consists of two stages: (a) the simple dissociation of the urea molecule into ammonia and free cyanic acid, and (b) the hydrolysis of the cyanic acid by the solvent [1913, 1918, 1920].



It has, of course, long been known that cyanic acid is formed when aqueous solutions of urea are heated above 70°, and advantage of this fact has been taken by Walker and Hambly [1895] to prepare pure silver cyanate, but the significance of this formation of cyanic acid has only recently been realised.

Up to the present, the Werner formula for urea has not been considered in connection with the enzyme decomposition of urea. If urease acts in the manner generally ascribed to catalysts and brings about zymolysis by accelerating greatly a normal chemical transformation, it seems probable that the mechanism of the decomposition of urea by urease is comparable with the decomposition of urea by acids, and by alkalis.

If this be so, a careful chemical examination of the system during zymolysis should reveal the presence of the products of dissociation.

THE UREA/UREASE SYSTEM.

In studying an enzyme system attention must be devoted to the following aspects: (1) the enzyme preparation, (2) the application of the enzyme, (3) the substrate, (4) the methods of analysis.

(1) *The enzyme.* A very satisfactory preparation of urease is obtained by extracting the fresh finely ground meal of soy-bean with ether in a Soxhlet extractor for six to twelve hours. By this means most of the fatty material is removed and a very active uniform dispersion is obtained by shaking up with water or with weak alcohol [Folin, 1919]. A better preparation may be got by extracting with absolute alcohol immediately after the ether treatment, but for the present work, attention was devoted to obtaining a standard preparation of uniform zymolytic properties rather than an enzyme preparation of approximate purity.

(2) *The application of the enzyme.* In a qualitative study of enzyme-action it is convenient to have some means of separating the enzyme from the main bulk of the substrate solution so that the subsequent analysis will not be complicated by the presence of enzyme material and debris. This was accomplished by enclosing the enzyme in a collodion tube in one group of the experiments, so that although the substrate had free access to the enzyme the latter did not escape into the bulk of the solution.

Several types of collodion tubes were examined. The most satisfactory ones were made by the Hatschek formula [1920]. The tubes were tested by dialysing urease solutions against distilled water and solutions of ammonium carbonate. Tubes that allowed sufficient enzyme material to escape in 24 hours at room temperature to give a positive biuret reaction on testing the surrounding fluid were rejected.

These collodion tubes are very permeable towards urea and ammonium salts.

In employing collodion separators in enzyme research it is necessary to consider the possible inactivation of the enzyme.

Urease suspensions of the strength used in the experiments were not found to be seriously affected by being kept in the tubes for periods up to two days. After that time, inactivation was apparent in the weaker suspensions, although this seemed to be not so much due to the loss of a dialysable component, or

"co-enzyme," as claimed by Onodera [1915], but rather to be an instance of membrane adsorption of the enzyme.

To obtain a large surface of enzyme suspension exposed to the substrate, a test-tube of smaller diameter was inserted into each collodion tube so that the urease occupied the space between the two tubes. Inverted specimen jars of capacity 100 to 1000 cc. with narrow necks were used to contain the substrate. The collodion tubes were of such size that they almost filled the necks of the jars.

The hydrogen ion concentration of the system was kept within the working range of the enzyme (*i.e.* p_H 6.8–7.2) by means of centinormal nitric acid and the use of a Cole-Onslow comparator.

(3) *The substrate.* The urea used was purified by two crystallisations from water, and was free from all cyanate, carbonate, carbamate, and ammonia reactions. Solutions of urea in water are readily attacked by the micro-organisms of the ordinary laboratory air, and, consequently, the substrate solutions were made up with sterile water and kept in clean stoppered bottles.

(4) *Methods of analysis.* The analysis of the products of urea decomposition in simple solutions is not a difficult matter, but when the solutions are liable to contain materials extracted from the enzyme special precautions have to be taken. Ammonia was detected by the usual Nessler method, and was estimated by aspiration and Nesslerisation or titration in a comparator.

The presence of traces of ammonia in the urease was of no consequence in most of the experiments. When necessary, it can either be estimated and allowed for, or removed by a few days extraction of the enzyme by absolute alcohol at 30°. Folin [1919] has advocated the use of "permutite" but the common fuller's earth seems to be effective also.

It may be noted, in passing, that solutions of urease will develop ammonia when kept for some hours in contact with strong alkalis at room temperatures.

Carbon dioxide was detected and estimated by aspiration into standard barium hydroxide solutions.

Cyanic acid was detected and isolated as the silver salt, by which means it has been found possible to separate it from carbonates and carbamates.

This separation is effected by adjusting the hydrogen ion concentration of the solution to p_H 5.0 by means of $N/50$ HNO_3 , and a comparator, using methyl-red as indicator. Under these conditions, all the silver carbonate (and carbamate, if any) is dissolved, and silver cyanate remains.

The presence of the cyanate radicle in the silver precipitate may be demonstrated by three colour tests.

For detecting small traces of cyanate the following method was devised. The silver precipitate, separated from carbonate and carbamate, is collected on a small filter paper and washed thoroughly with distilled water until the washings no longer give any ammonia reaction with Nessler's reagent. The precipitate on the paper is treated with a few drops of warm $N/2$ HCl ; this liberates and hydrolyses any cyanic acid present, and on washing the

precipitate once more, the filtrate gives a positive Nessler reaction. This is an exceedingly delicate test for cyanates, provided proper precautions are taken to see that the reagents are ammonia-free. The cyanate radicle may also be demonstrated by treating the precipitate with a few drops of 5 % hydroxylamine sulphate, and after a couple of minutes adding a little dilute ferric chloride; a purple colour is produced. This test is due to Hantzsch and Sauer [1897]. The colour is due to the liberation of cyanic acid and the combination of this with hydroxylamine to form a hydroxy-urea, the ferric salt of which is dark purple in solution. It has been developed by the present author into a method for estimating cyanates colorimetrically. Certain precautions must be taken in applying the test to traces of cyanates. The presence of much ammonia interferes with the test by obscuring the characteristic black-purple shade. The hydroxylamine must remain in contact with the free cyanic acid for sufficient time to interact to form the hydroxy-urea. In the presence of excess of the hydroxylamine the coloration is not permanent as the ferric salt is soon reduced to an almost colourless ferrous salt. The colour can be restored by careful addition of more ferric chloride or by adding a drop of hydrogen peroxide solution (which must be free from acetanilide, or any other preservative which gives a colour with ferric salts). Once the solution has been freed from uncombined hydroxylamine the colour is permanent.

Cyanates formed in solution may also be identified by the fact that the free cyanic acid combines with urea to form biuret.

Cyanates in quantity can be most rapidly shown (as, for example, the sublimate formed by heating dry urea in a test-tube) by the intense blue colour given with solutions of cobalt acetate or nitrate.

The delicacy of these tests under general conditions is as follows. Nessler method, 1 in 400,000; hydroxylamine reaction, 1 in 2000; cobalt method 1 in 90; but the delicacy can be greatly increased by using the centrifuge to collect the silver precipitates and by careful washing with ice-cold water.

Biuret was identified and estimated colorimetrically by the copper method, as described by Werner [1913], under conditions when the absence of foreign protein material derived from the enzyme was assured.

These analytical methods are grouped here for convenience, as they will be referred to in subsequent papers.

Experimental. Series I. Detection of cyanates in the urea/urease system.

Fresh solutions of pure urea in distilled water saturated with CO_2 were prepared of concentrations 1, 5, 10, 20 %, and submitted to the action of freshly extracted urease (1 g. in each collodion tube). The solutions were kept at low temperature, $1^\circ\text{--}5^\circ$, to retard hydrolysis of the cyanic acid. At hourly intervals samples were drawn off from the outer containing jars, treated with $N/10 \text{ AgNO}_3$, the solution set to $p_{\text{H}} 5$ by $N/50 \text{ HNO}_3$, and filtered through a close filter paper.

After two hours all the solutions gave positive reactions for cyanic acid by the Nessler method, this continued for several hours in the solutions, but entirely disappeared from the 1 % solution on keeping the flasks at room temperature overnight. All the solutions next day gave a definite biuret reaction, most marked in the strongest solution of urea. By means of a control it was found that this did not arise from the escape of protein material from the dialysing tubes, but was due to the interaction between cyanic acid and the substrate.

Cyanic acid was also shown in solutions of urea undergoing zymolysis in ordinary flasks in direct contact with the enzyme, by treating samples drawn off from time to time with excess of colloidal ferric hydroxide, which precipitates nearly all the ammonia from the solution and brings down the enzyme with the precipitate. On centrifuging the samples, cyanates were found to be present in the clear supernatant layer by applying the hydroxylamine test, and by pipetting off the clear solution and applying the silver method.

The conclusion from a large number of experiments of this type is that cyanic acid is produced during the decomposition of urea by urease. The yield of cyanic acid as silver cyanate in these experiments was always small; up to 0.1 g. from a litre of 5 % urea solution being obtained.

Experimental. Series II. Estimation of the cyanic acid formed during the zymolysis of urea.

Method A. Substrate, 5 % urea. Enzyme, 5 % extracted urease suspension which had been kept for three days at 40°, until the coarser particles had subsided. Samples of 20 cc. of substrate and 10 cc. of enzyme suspension were mixed at 40°, set to p_H 6.5, and incubated at 40°.

At hourly intervals, duplicate pairs of flasks were withdrawn and each treated with 1 cc. of $N/1$ $AgNO_3$. One flask was then titrated to p_H 5 with 0.6 N HNO_3 , using methyl-red paper¹ and a standard flask for comparison.

The acid used gave a measure of the relative degree of zymolysis, and also dissolved any silver carbonate or carbamate that might be present, leaving silver cyanate, silver chloride and precipitated enzyme.

The contents of the other flask were then treated with the same amount of 0.6 N HNO_3 , to bring it to p_H 5 using no indicator. Both samples were then filtered and the precipitates washed three times with a fine jet of cold distilled water. The silver in the filtrate was determined by titration with thiocyanate of such strength that 1 cc. corresponded to 0.015 g. of potassium cyanate. The difference between these values and the value of the silver originally added represented the silver left on the filter paper. By titration, the amount of silver nitrate combining with 10 cc. of the uniform enzyme suspension was determined under comparable conditions, and the difference

¹ Methyl-red paper was used in preference to the solution, so as to have a colourless liquid for the subsequent titration.

between the amount of silver required to precipitate the enzyme together with associated inorganic salts, and the amount of silver left on the filter paper gave the silver fixed as silver cyanate. The presence of cyanic acid in the silver residue was confirmed by the tests described.

The enzyme suspension employed was sufficiently uniform to give an almost constant silver value in a series of six controls.

The chief error was found to lie in the washing of the precipitate on the filter paper. If it is washed for some time with distilled water at room temperature the silver gets ultimately washed away from its union with the protein, but by adopting a uniform technique this error was rendered negligible.

Table I.

Time in hours	Percentage urea decomposed	Percentage cyanic acid present
0	0	0
1	11.16	Not estimated
2	24.84	0.007
3	30.78	0.009
4	32.23	0.011
5	35.98	0.010
7	45.00	0.010

Temperature, 40°.

Enzyme, 10 cc. uniform suspension of urease decanted from 5 % suspension.

Substrate, 20 cc. 5 % urea.

Initial p_H 5.5.

Here it will be seen that the concentration of cyanic acid rises to a maximum and remains remarkably constant. The concentration will obviously depend on the difference between the rate of formation and the rate of removal by hydrolysis of the cyanic acid. When this difference in the rates is constant the concentration of cyanic acid will be constant. Since cyanic acid is very readily hydrolysed in weak aqueous solution at room temperature, it might be expected that the lower the temperature of the urea solution undergoing zymolysis the higher would be the concentration of cyanic acid, since it would accumulate in the solution. A series of experiments showed that this is not so. The amount of cyanic acid in solution is a function of the rate at which the urea is being decomposed, and this decomposition has a higher temperature coefficient than has the decomposition of the cyanic acid by hydrolysis.

To reduce as far as possible the errors introduced into the estimation by the presence of excess of urease material a method was devised whereby the cyanic acid could be estimated in solutions free, or almost free, from enzyme.

Method B. Estimation of cyanic acid. The urease was enclosed in a collodion sac and the cyanic acid was estimated in samples drawn from the surrounding solution. The proportions were 10 cc. of 10 % urease solution in the sac to every 100 cc. of 2 % urea solution in the containing vessel. At definite intervals 20 cc. samples were drawn off in duplicate, set to p_H 5.0 using 0.7 *N* HNO_3 , and methyl-red paper. One sample was boiled for half a minute to destroy any cyanic acid present, then each sample was treated with an excess of

$N/10$ $AgNO_3$, filtered, and the silver estimated in the filtrate. The difference in the silver value gave the amount of cyanate present. The substrate and the enzyme in these experiments were in the ratio of 100 cc. 2% urea to 10 cc. 2% urease.

Table II.

Temperature of experiment	Maximum percentage of cyanic acid
2-3°	0.0016 after 45 hours
5°	0.0043 " 30 "
7.5°	0.0034 " 20 "
10°	0.0301 " 24 "

At temperatures above 10° the collodion sacs became very fragile. In general, the lower the temperature the longer the cyanic acid took to attain its maximum value. In the experiments carried out at a temperature just above freezing point, the cyanic acid attained a concentration of 1.6 mg. per 100 cc. substrate when 5.01% of the total urea had been decomposed. On leaving the solution at 5° for another period of 15 hours the concentration of the cyanic acid had fallen to 0.3 mg. per 100 cc. owing to its slow hydrolysis in the alkaline liquid.

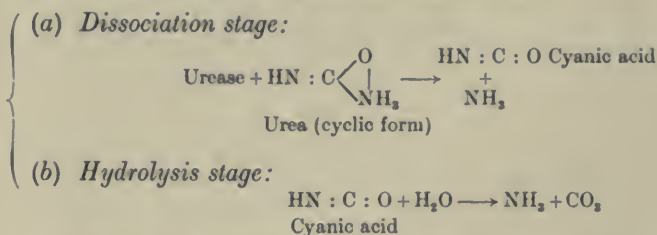
Significance of the presence of cyanic acid in the urea/urease system.

Cyanic acid may represent the intermediate stage in the zymolysis of urea, or may be the result of some comparatively unimportant side reaction. Since the acid is not formed from sterile solutions of urea at the temperature of the experiments, it must be produced by the action of the enzyme on the urea (urease alone, of course, contains no cyanates nor gives rise to them in simple solution). From what is known of the part played by this substance in the normal decomposition of urea, it is probable that it is the true intermediate compound in the enzyme decomposition of urea.

Up to the present, Yamasaki [1918, 1920] appears to be the only worker who claims to have detected ammonium carbamate in the urea/urease system. Unfortunately, his results can hardly be considered conclusive since the method he adopts for estimating carbamate is equally applicable to cyanate. Yamasaki, working on the basis of the old "carbanide" formula for urea, does not appear to have considered cyanic acid at all in connection with the change, which he ascribes to a successive hydrolysis of the urea by simple catalytic action in the substrate—an explanation that affords no light on the mechanism of the process.

By the methods described in the present paper, cyanic acid can be detected and isolated as the silver salt in the presence or absence of carbamates, and, while there is no experimental evidence that ammonium carbamate is not formed during the enzyme decomposition of urea, there is evidence that cyanate does occur and that the decomposition of urea by urease proceeds along the same chemical lines as the normal decomposition of urea in solution by acids, alkalies, or the action of heat.

The change may be represented as follows:



Urease is not directly concerned in the "hydrolysis" of urea. The function of the enzyme is to bring about dissociation of the urea into ammonia and cyanic acid. The hydrolysis of the cyanic acid follows as a secondary change in the presence of water. This has been supported by observations on the action of urease on solutions of urea in alcohol, where the cyanic acid could not undergo hydrolysis.

It will be shown that this conception of urease as a *dissociating* enzyme and not a *hydrolysing* enzyme provides a new light for the investigation of enzyme mechanism, and accounts for, amongst other things, the specific action of the ferment.

CONCLUSIONS.

1. Cyanic acid has been isolated as the silver salt from solutions of urea undergoing decomposition by urease.
2. Cyanic acid attains to a maximum concentration in the urea/urease system, and is being continually produced as fast as it is removed by the hydrolytic action of the solvent.
3. Cyanic acid and ammonia are the intermediate products in the enzyme decomposition of urea.
4. The enzyme decomposition of urea proceeds along the same lines as the normal decomposition of urea in solution by acids, alkalies, or heat.
5. Urease is a *dissociating* enzyme; decomposing the neutral urea molecule into an alkaline component, ammonia, and an acid component, cyanic acid, which is rapidly hydrolysed by the solvent into ammonia and carbon dioxide.

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XIII. THE STEROL CONTENT OF COW'S MILK.

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THE following account of the nature and composition of the so-called "unsaponifiable matter" found in milk fat, originated from analyses required for some experiments on cholesterol metabolism in adults. Ordinary cow's milk contains nearly one-tenth per cent. of a mixture of substances which can be extracted with ether or light petroleum after saponification of the fat by alkalis, and after evaporation of the ether is obtained as a soft golden yellow solid. In spite of the large number of investigations that have been made upon milk fat, this fraction has been invariably dismissed, until recently, by saying that it consisted of traces of cholesterol, wax, colouring matter etc.; this was no doubt largely due to the lack of any good method of separation or estimation of the constituents.

In 1883, Schmidt-Mülheim [1883] drew attention to a substance present in milk which he considered to be cholesterol on account of its colour reaction. Menozzi [1903] was perhaps the first to investigate the substance more closely and he showed that the sterol was identical in physical and chemical properties with the cholesterol isolated from bile. The earlier quantitative estimations of the cholesterol content of milk were made by Magnus-Levy [1910], Tolmatscheff [1867], Siegfeld [1906], Grigaut [1913], and others. Their values range from about 0.01 to 0.04 g. per 100 cc. milk.

In 1918 Denis and Minot [1918] published a series of analyses both of cow's and human milk made by Bloor's colorimetric method [1916]; this method however is known to give rather too high results for some tissues. Their figures for the fat and total cholesterol in 100 cc. of cow's milk are given in Table I.

Table I.

Fat	5.0	4.6	4.4	4.2	4.7	3.4	5.3	3.4
Cholesterol	0.0176	0.0176	0.0164	0.0162	0.0158	0.0156	0.0152	0.0152
Fat	4.0	3.9	3.3	4.5	3.7	3.4	3.2	
Cholesterol	0.0144	0.0140	0.0136	0.0128	0.012	0.0112	0.0105	

Average fat, 4.07.

Average cholesterol, 0.0145.

The average percentage of cholesterol per 100 g. of fat is thus 0.356. Last year, while our work was in progress, Wacker and Beek [1921] published a series of determinations made by the digitonin method of Windaus, which are summarised in Table II.

Table II.

Fat in 100 cc. milk	4.02	4.00	3.75*	3.72	3.69	3.46	3.31	3.31
Cholesterol in 100 cc. milk	0.014	0.014	0.016	0.012	0.011	0.012	0.011	0.011
Cholesterol in 100 g. fat	0.351	0.352	0.424	0.315	0.300	0.347	0.329	0.327

Averages: 3.65; 0.0126; 0.343.

The work to be described in the present paper may for convenience be divided into three parts: (1) quantitative estimation of cholesterol content of fresh and condensed milk; (2) qualitative examination of the fraction of the sterols precipitable by digitonin; (3) separation and quantitative examination of the residual non-precipitable oils.

METHOD OF ANALYSIS.

All our analyses have been made on the fat as separated by the well-known Gottlieb-Rose method.

The fat was divided into two portions, one for determination of the free and the other for determination of the total free and combined cholesterol. The estimation was made by the method described by Fraser and Gardner [1910] with some slight modifications, which we detail briefly. To estimate the total free and combined cholesterol the fat was dissolved in ether and a large excess of a solution of sodium in 97 % pure alcohol, containing about 8 to 10 times the theoretical quantity of sodium required, added. This mixture was allowed to stand 24 hours, and then heated under a reflux on the water bath for 4 to 5 hours, in order to ensure the complete hydrolysis of cholesterol esters. It was then largely diluted with water and repeatedly extracted with ether until nothing more was dissolved. The ethereal solutions were united, thoroughly washed by shaking with water until quite free from traces of soap and made up to known volume. A suitable aliquot portion was then evaporated until free from ether, dissolved in alcohol and precipitated with a considerable excess of a 1 % alcoholic solution of digitonin. After standing overnight the alcohol was completely evaporated at the lowest convenient temperature. The residue was washed by decantation with ether to remove unprecipitated oily matter, then with warm water to remove excess digitonin. Finally the precipitate of cholesterol-digitonide was brought on to the Gooch, the washing completed and the substance dried and weighed.

For estimation of free cholesterol the fat was dissolved in alcohol and precipitated directly with excess of 1 % digitonin solution. In this case it was found advantageous not to evaporate the alcohol completely, but to leave

about one-fourth of the total volume, otherwise it is sometimes difficult to wash away all traces of fat and colouring matter with ether. After filtering off the alcohol and washing the precipitate with ether the rest of the procedure was as described above.

Fresh milk.

For fresh milk we used two samples of "Grade A certified milk," as defined by the milk section of the Ministry of Health, and two samples of so-called "Nursery Milk" stated to be obtained from a herd of tuberculin tested cattle which yield a milk higher than the average in butter-fat. The results are given in Table III.

Table III.

No.	Description	Fat per 100 cc. g.	Unsaponifiable matter in 100 cc. milk g.	Free cholesterol per 100 cc. g.	Ester cholesterol per 100 cc. g.	Total cholesterol per 100 cc. g.	Total cholesterol in 100 grams of fat g.
(I)	Nursery milk (1922)	4.46	—	0.001	0.015	0.016	0.358
(II)	" " "	4.14	0.0725	0.0144	0.0029	0.0173	0.418
(III)	Grade A (1922)	3.38	0.082	0.0114	nil	0.0114	0.334
(IV)	" "	3.47	0.066	0.0113	0.0044	0.0157	0.344
	Mean	3.86	0.0735	0.0095	0.0056	0.0151	0.3635

Condensed milk.

In order to cover a wider range of conditions, we also examined a number of samples of condensed milk. These were ordinary shop samples, described as consisting of whole milk, and had been prepared in England, United States, Norway, Denmark and Switzerland. The results are given in Table IV.

Table IV. *Condensed milk, bought 1920 and 1921.*

Description	Specific gravity	Total solids per 100 g. milk	Ash per 100 g. milk	Protein total N $\times 6.38$ per 100 g.	Fat	Total unsaponifiable matter per 100 g. milk	Total cholesterol free and ester per 100 g. milk	Total cholesterol per 100 g. milk	Unsaponifiable matter not precipitated by digitonin per 100 g. milk fat	Remarks
(1) Unsweetened	1.085	28.12	1.43	7.44	8.47	0.118	0.0336	0.401	1.000	} same makers
(2) Sweetened (a)	1.284	80.52	2.14	9.77	10.50	0.133	0.0268	0.255	1.010	
" (b)	—	—	—	—	—	0.145	0.0227	0.216	1.060	
(3) Unsweetened	1.074	25.80	1.38	7.12	7.98	0.062	0.0227	0.256	0.529	} same makers
(4) "	1.091	32.68	1.63	8.38	10.12	0.067	0.0317	0.314	0.329	
(5) Sweetened	1.294	74.45	1.60	7.70	9.32	0.082	0.0310	0.333	0.542	
(6) Unsweetened	1.072	25.77	1.32	6.61	8.34	0.125	0.0428	0.513	0.990	
(7) Sweetened	1.309	75.58	1.70	7.33	8.65	0.161	0.0486	0.562	1.298	
(8) "	1.288	73.20	1.78	7.60	9.30	0.053	0.0248	0.256	0.314	}
(9) Unsweetened	1.073	26.40	1.47	7.05	8.22	0.044	0.0201	0.245	0.293	
(10) Sweetened	1.308	76.16	1.81	8.03	8.96	0.055	0.0266	0.295	0.335	
(11) "	1.305	77.23	1.94	8.78	10.89	0.085	0.0317	0.291	0.490	}
	Mean				9.16	0.094	0.0303	0.328	0.691	

For the sake of comparison, the average results of various observers are brought together in Table V.

Table V.

	Fat per 100 cc. milk	Cholesterol per 100 cc. milk	Cholesterol per 100 g. milk fat
Denis and Minot	4.07	0.0145	0.356
Wacker and Beck	3.65	0.0126	0.343
Fox and Gardner (fresh milk)	3.86	0.0151	0.364
.. .. (condensed milk)	—	—	0.339
Mean	3.86	0.0141	0.351

It will be seen from the above tables that the percentages of cholesterol found by the various methods quoted are in fairly close agreement.

Ratio of total cholesterol to fat.

The most obvious preliminary point to settle seems to be whether there is any definite relation between cholesterol and fat content. The average figures in Table V would at first sight indicate that there is a rough proportionality. This has already been pointed out by Denis and Minot, and their table, quoted above, which is arranged in order of descending values of cholesterol is suggestive, although there are several quite marked exceptions. Neither the figures of Wacker and Beck nor of ourselves however point to any exact ratio. It may also be mentioned that no such exact proportionality is found in tissues, blood etc. It will be noticed that the condensed milks show a much greater variation in the percentage of cholesterol in the fat than the specimens of fresh milk examined. Assuming that these variations are real and do not merely result from the removal or addition of cream or from the various processes of manufacture employed, they may perhaps be accounted for by differences of breed, feeding, climate, etc.

Breed. The percentage of fat varies very markedly with the breed of cow. The extent of the variation is well shown by the following figures, compiled by Wing [1897] from a large number of analyses.

Breed:—	Friesian- Holstein	Shorthorn	Ayrshire	Jersey	Guernsey
Average % of fat in milk	3.51	3.65	3.68	4.78	5.02

In actual practice the range may be considerably higher as it is not very uncommon for a Friesian cow to give milk with a fat percentage below 3, and for a Guernsey to reach 6 or even 7 %. In this connection however it must be remembered that the total volume of milk produced also varies and in the opposite sense, so that the actual output of fat in a given period according to Henry [1911] is nearly the same for all dairy breeds.

In order to test the effect of breed on the fat and cholesterol content of the milk we secured two samples, one from a Friesian cow known to yield a milk low in fat and a second from a good average Jersey. The analyses are given in Table VI.

Table VI.

Breed	Fat per 100 cc.	Cholesterol per 100 cc.		Total	Total cholesterol per 100 g. fat
		Free	Ester		
Friesian	2.95	0.0096	0.0023	0.0119	0.404
Jersey	5.96	0.0180	0.0047	0.0227	0.381

Here the output of cholesterol appears to follow approximately the output of fat.

Diet. Of the influence of this factor upon the amount of cholesterol in milk little can be said with certainty. Generally speaking, diet, if sufficient, is stated to have little effect on the milk, either quantitatively or qualitatively. Thus it appears to be easier to increase the yield by constant, regular and complete milking than by increased or richer feeding. What would be the effect of a prolonged period of more or less deficient diet on the fat-cholesterol content of the milk we do not know, but it is perhaps not without significance that three samples of milk, two purchased in London and the other in Cambridge for metabolic experiments in the summer of 1917 and early spring of 1918, gave the following results.

	Fat per 100 cc.	Total cholesterol per 100 cc.	Total cholesterol per 100 g. fat
(1)	3.73	0.007	0.188
(2)	3.50	0.007	0.200
(3)	2.92	0.009	0.308

It is well known that certain feeding stuffs such as cotton and linseed meal have an effect on the physical character of the fat. Further in a series of interesting experiments Palmer and Eckles [1914] were able to show that almost colourless butter can be obtained if the diet is kept sufficiently free from the lipochrome carotene. Here also the shortage is at first made good by reserves in the tissues; this is the probable explanation of "pale" winter butter sometimes met with. Apparently we have here a case of one constituent of milk being directly dependent for its supply upon the diet. Whether the same applies to the sterol there are no data to say, but experiments are in progress which we hope may throw light on this point.

Lactation period. As the lactation advances some changes in the fat content of milk occur, but we do not know whether the sterol follows suit. We have not had an opportunity of examining cow's colostrum from this point of view, but we agree with Wacker and Beck in finding that human colostrum is much richer in cholesterol than normal human milk. This however we propose to deal with in another communication.

Proportion of free cholesterol to cholesterol in form of esters.

According to Wacker and Beck cholesterol is not found in the free state in milk but in the form of esters, presumably esters of oleic and palmitic acids. This conclusion was based on the following experiment. Milk fat was shaken in a separating funnel with hot alcohol. Since free cholesterol is easily soluble

in hot alcohol, while the oleic and palmitic esters are insoluble or difficultly soluble, they expected that any free cholesterol would separate out from the alcohol solution in characteristic crystals, or could be detected by the digitonin reaction. They were unsuccessful in finding free cholesterol by this method. They assumed however without much justification that the presence of fat would not influence the solubility in alcohol. Our own conclusions are almost exactly the opposite. As will be seen from Table III, in all except the first case we found that the main proportion was in the free state. The average ratio of free to ester cholesterol was 1 : 0.54. In the case of the Friesian and Jersey cows (Table VI) the proportion of free to ester cholesterol was in the first case 1 : 0.24 and in the second 1 : 0.26.

Distribution of cholesterol in milk.

Little is known of the manner in which the cholesterol is distributed throughout the milk. Being quite insoluble in water it might be supposed that it was merely dissolved in the globules of fat, but as Wacker has already pointed out this scarcely explains the facts. He finds that the liquid which remains after the fat has been removed either by the separator or during churning is richer in cholesterol than can be accounted for on this hypothesis by the small amount of fat still remaining. According to his analyses the fat in skim milk would contain 0.80 % of cholesterol instead of 0.38. We came to the same conclusion in another way since we noticed that butter fat invariably shows a lower percentage of total cholesterol than the whole milk fat. This difference will be seen from a comparison of the average percentage cholesterol content of milk fat—0.35 in Table IV with the following analyses of samples of genuine butter given in Table VII.

Table VII.

	Total cholesterol %
(1) Reading Agricultural Dept. College Farm herd	0.278
(2) Butter made from milk of Jersey cow	0.291
(3) Good commercial sample	0.225
(4) Commercial sample	0.254
Mean	0.244

In order to confirm this we made the following analyses of samples of separated milk and butter milk kindly procured for us by Lord Rayleigh Dairy Co. (Table VIII).

Table VIII.

	Fat per 100 cc.	Cholesterol per 100 cc.		Total	Percentage of total cholesterol in fat
		Free	Ester		
Skim milk	0.187	0.0029	0.0008	0.0037	1.912
Butter milk	0.511	0.0089	0.0012	0.0101	1.977

These facts rather suggest that while no doubt most of the cholesterol—free and ester—is present dissolved in the fat globules, a certain amount may be present in some other form, probably in colloidal solution, or possibly even

combined with the protein. We have found a somewhat similar state of things when examining ascitic fluids, which, even when apparently clear and capable of being filtered through paper without any residue, often contain quite considerable quantities of cholesterol and cholesterol esters.

QUALITATIVE EXAMINATIONS OF THE STEROLS OF MILK.

The sterol which can be precipitated by digitonin, as will be seen from the tables, forms only about one-third to half of the total unsaponifiable matter of the fat, the remainder consisting of sticky yellow oils, very inactive and not easy to deal with or characterise.

In order to examine both precipitable and non-precipitable portions more closely we isolated a considerable quantity, using butter as a convenient source. The bulk of the fat was removed by saponification with caustic soda and the unsaponified portion was subjected to a final saponification by means of sodium ethoxide in alcoholic solution, the mixture being boiled for several hours, in addition to standing 24 hours, in order to ensure decomposition of difficultly hydrolysable sterol esters.

The yield of unsaponifiable matter from butter is somewhat lower than from milk since as mentioned above some sterol remains behind in the butter milk. A further minor source of error, which accentuates this difference between the percentage of unsaponifiable matter in milk fat and butter fat, is due to the small amount of resinification which takes place on hydrolysis by alkalis in alcoholic solution [Gardner and Fox, 1921]. This error is proportionally greater when small quantities of fat are analysed, so that the figures given in the tables for unsaponifiable matter are probably rather too high.

Sterols precipitated by digitonin.

The crude sterol was separated as far as possible from oily matter by crystallisation from acetone or alcohol, and the last traces removed from the oils by precipitation with digitonin. The digitonide was filtered off and decomposed as recommended by Windaus by heating in the vapours of boiling xylene. The whole crystalline matter was now subjected to a thorough fractional crystallisation, melting points taken and the benzoates of several fractions prepared. All the fractions corresponded in properties to cholesterol and cholesterol only. The final residues, too small to purify by further crystallisation, were benzoylated by the pyridine method, but again the result was a typical cholesterol benzoate.

We therefore conclude that the precipitable portion of the unsaponifiable matter of milk fat consists entirely of cholesterol.

Oils not precipitated by digitonin.

We were not successful in isolating any pure substance from these oils, but they appeared to consist in part at least of stable esters which escaped saponification by alkali. We attempted to purify the oils by distillation in

super-heated steam. This helps to remove resinous matter, but also appears to hydrolyse some of the very stable esters which occur in these mixtures. The distillation proceeded very slowly and there was not quite the same tendency for the distillate to assume the characteristic candle-like solid emulsion noted in the case of similar faecal oils [Gardner, 1921]. The distillate was dissolved in ether and the ethereal solution shaken out with alkali. From the alkaline solution a small quantity of a crystalline acid was obtained, which was purified by distillation *in vacuo*. It had no definite melting point and on titration with alkali proved to have an average molecular weight of about 288. The neutral portion after evaporating the ether was distilled in a high vacuum. It passed over between 170–180° for the most part. An attempt was made to fractionate this and eventually two main fractions were obtained: (1) distilling up to 175° (2 mm.) and (2) above 175°. Fraction (1) was a mobile golden yellow liquid at ordinary temperature, but solidified in a freezing mixture. It gave on treatment in chloroform solution with acetic anhydride and a drop of sulphuric acid a dark brown colour which more or less rapidly changed to a dusky olive green very similar to the colour obtained with faecal oils [Gardner, 1921]. On combustion 0.1885 gave 0.2049 H₂O and 0.5629 CO₂. C = 81.44 %, H = 12.08 %.

The fraction 2 was again hydrolysed, this time by heating in a sealed tube at 110° with alcoholic potash. The liquid was diluted and extracted with ether and the ethereal solution shaken out with alkali as before. A very small fraction of acid, apparently a mixture of a liquid and a solid acid was obtained. This was distilled *in vacuo* and by titration with alkali an average molecular weight of about 274 was indicated. The acids were too small in amount for identification, but the liquid portion appeared to be unsaturated.

The neutral portion after removal of the ether distilled in a high vacuum at about 200°. It came over as a clear golden yellow amorphous glass which just melted on a warm day. On freezing out from acetone a trace of a white wax-like substance was obtained too small however for further purification. On combustion of the amorphous glass 0.1543 gave 0.1579 H₂O and 0.4582 CO₂. C = 81.00 %, H = 11.37 %.

On testing an alcoholic solution of this oil with digitonin a mere trace of a digitonide was precipitated, far too small in quantity however to do anything with.

We take this opportunity of thanking Capt. Golding, of University College, Reading, and Miss E. Cornish, of Steyne, for kind help in obtaining some of the samples of milk and butter required, and also the Government Grant Committee of the Royal Society for help in defraying some of the cost of the work.

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XV. NOTE ON THE SULPHURIC ACID TEST FOR LIVER OILS.

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(Received January 15th, 1923.)

THE well-known test for liver oils, which consists in the production of a blue or purple coloration on agitating a solution of the oil in carbon disulphide or chloroform with a drop of concentrated sulphuric acid has generally been attributed to the presence in such oils of cholesterol and lipochromes. Recently, however, Drummond and Watson [1922] have drawn attention to the parallelism occurring in a long series of oils between the production of this coloration and the presence of vitamin A, and they conclude that the substance, to which the formation of the coloured compound is due, is neither cholesterol nor, probably, a member of the lipochrome pigments. The general resemblance between this test and that of Pettenkofer for bile acids or Mylius's modification, in which the sugar is replaced by a solution of furfuraldehyde, suggested to us that the reaction might be of the same type as in these latter, namely, a condensation between two compounds under the influence of sulphuric acid. Cholesterol might thus be involved in the reaction though not of itself capable of giving the test, while the second substance, which must also be present in the oil, might be a derivative of furfuraldehyde or other compound of similarly reactive nature. Such a reaction occurs in Neuberg and Rauchwerger's [1904] test for cholesterol in which δ -methylfurfuraldehyde, formed from rhamnose, is employed, a red coloration being produced under the influence of concentrated sulphuric acid.

The purple coloration given by liver oils can, we find, be closely simulated by adding a drop of concentrated sulphuric acid to a light petroleum solution containing both cholesterol and furfuraldehyde or ω -hydroxymethylfurfuraldehyde. Chloroform may also be used as a solvent but unless the solutions are carefully dried, several drops of sulphuric acid will be required to produce the coloration. Further, the addition of furfuraldehyde to such oils as butter, which of themselves give only a faint coloration, causes the production of a very intense purple colour on adding the sulphuric acid.

So far all attempts to obtain furfuraldehyde, or some compound which could replace it in this reaction, from coal fish oil by distillation with steam or under reduced pressure and in other ways have been unsuccessful.

The reaction provides a delicate test for cholesterol, a faint purple colour being slowly given by 0.1 mg. in 5 cc. light petroleum containing excess of furfuraldehyde, while with 0.5 mg. cholesterol the coloration is immediate and pronounced.

Since these experiments were carried out a paper by G. S. Whitby [1923] has come to our notice, in which somewhat similar tests for cholesterol using formaldehyde are described and in view of this we do not propose to continue the work from this aspect.

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Neuberg and Rauchwerger (1904). *Chem. Centr.* **2**, 1434.
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XVI. ESTIMATION OF SUGAR IN THE BLOOD.

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(Received November 15th, 1922.)

FOR some years past much attention has been directed to the determination of carbohydrate tolerance by observing the variations that occur in the sugar-content of the blood after the ingestion of a known amount of sugar. The value of this method is now universally recognised. It is not intended in this paper to treat the subject in a general way, but merely to consider the actual estimation of sugar as required for the above purpose.

The usual procedure is to determine, first of all, the percentage of sugar in the blood at the "fasting level," then to administer a dose of 30-50 g. of glucose and make further estimations, say, every 20 to 30 minutes for two hours.

Micro-methods are practically always employed, the blood being obtained from a prick of the finger. After trying a number of these methods, it was found that those of Bang, MacLean, Folin and Wu, and Mackenzie Wallis and Gallagher were the most satisfactory. Considerable time and labour are required, however, for the routine performance of any one of these.

For some time past the writer has been endeavouring to simplify existing methods, especially from the clinical standpoint, and to increase, if possible, their accuracy. After a number of investigations, it was finally decided to use the rationale of the method of Folin and Wu [1918, 1920], and Mackenzie Wallis and Gallagher [1920] as a basis for this purpose. The process evolved has now been in use for some time and has proved to be most satisfactory. It differs in so many points from the original—in the collection and dilution of blood, the type of standard colour, the size of the boiling-tube, the curve of correction for copper reduction, etc.—that a full description is necessary.

Method of obtaining blood.

An amount of blood sufficient for accurate estimation of the sugar-content can be obtained from a single prick of the finger. The blood is collected in a small platinum capsule, specially designed for the purpose. This has been found to be the simplest and most efficient way of securing the sample. The collection is made with the greatest ease and the sample of blood is greater in amount than that which can be conveniently obtained by the blotting-papers in general use. It is considered preferable to weigh the small quantity of blood on a torsion balance, than to measure it in a pipette.

Pipettes must be kept scrupulously clean, otherwise blood will probably not run into them. The use of the capsule avoids this trouble.

The platinum capsule is held by a small pair of cross-action forceps (both being shown full size in Fig. 1), and is then hung on the hook of the torsion balance and weighed.

It has been found throughout many blood-sugar estimations, that the weight of the capsule has not varied in the slightest degree; numerous weighings can therefore be omitted. A capsule weighing about 170–200 mg. has proved most suitable.

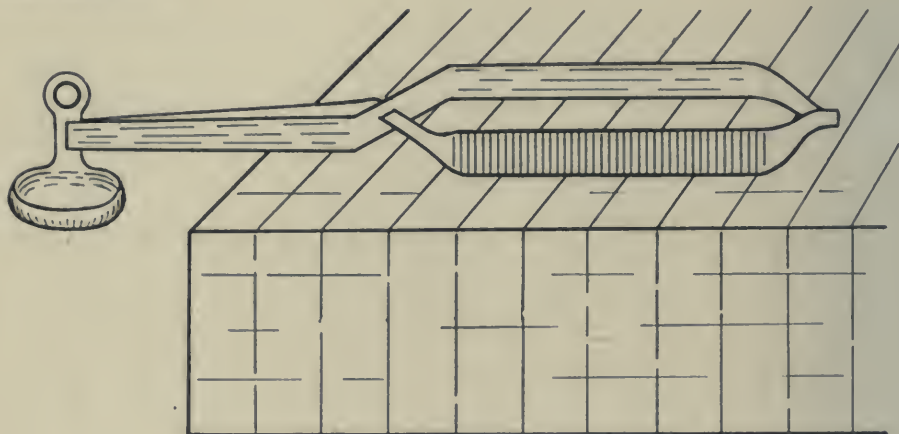


Fig. 1.

The hand of the patient is washed in warm water and dried. Then the thumb (for instance) is cleansed with ether. The patient is instructed to swing the arm, keeping the hand as low as possible and further congestion is caused by winding at once a piece of rubber-tubing round the proximal part of the digit. A prick is made with a bayonet-pointed needle to the side of, and just above, the nail. The thumb is then turned over and the blood made to fall in drops *directly* from the point of puncture into the capsule (held by the forceps) by compression of the pulp and nail. The capsule and blood are weighed immediately. One should obtain 200, or more, mgm. blood; generally it is quite easy to collect up to 270 mgm. It has been found necessary to emphasise: (a) that the hand should be warm and (b) the thumb thoroughly congested in order to secure the sample in the most convenient way. *Without delay* the capsule and blood are dropped into a short, wide test-tube (see list of apparatus) containing 7.3 cc. of distilled water. A turn or two of the test-tube is sufficient to mix completely the blood and water; none of the blood clings to the capsule.

The lengthy process of sugar extraction, required in blotting-paper methods, is, of course, unnecessary here. In a couple of minutes the mixture becomes clear and then one proceeds immediately to remove the proteins and other interfering substances by using the tungstic-acid-precipitation method of Folin

and Wu. Accordingly 0.3 cc. of 10 % sodium tungstate solution is added and directly afterwards 0.3 cc. of $\frac{2}{3}$ N sulphuric acid. This makes the volume up to 8 cc. (0.1 cc. being allowed for blood, less evaporation during expt.). It should be well mixed to ensure full precipitation; a reddish-brown or chocolate-coloured precipitate is quickly formed. The whole is now filtered through starch-free filter paper, the No. 30 Whatman filter paper being the best for the purpose. By pouring in about half of the solution and waiting until the paper is completely moistened before adding the remainder, one obtains the maximum amount of absolutely protein-free filtrate. The filtrate is watery-clear, and from it one determines the percentage of sugar in the blood.

Principle of method of estimating sugar content of blood.

As in the other methods mentioned, the power of glucose to reduce an alkaline solution of copper is utilised. By the addition of phospho-molybdic acid to the cuprous oxide so produced, a clear deep-blue solution is obtained, any unreduced copper being at the same time decolorised. The depth of colour is a measure of the amount of reduction and consequently of the percentage of sugar present. This estimation is made by comparison in a colorimeter with the depth of colour similarly produced from a solution of glucose of known strength. A special feature, however, of the present method of sugar estimation is the simplification of the standard used.

The standard colour used in the colorimetric estimation of sugar.

The present method of producing the standard colour is a fixed procedure requiring frequent repetition. To simplify matters it was decided to replace the standard solution by a glass, or glasses, of exactly the same shade of colour. This was easily accomplished and a perfect matching of colours obtained. The colorimeter reading gave the value of the glass in terms of sugar solution. It is evident that one can afford to devote much more time and care to this single determination than to the routine preparation of standards, which are so liable to vary one from another. Several readings of the colorimeter ensure that an accurate value has been obtained for the particular glass used. In the construction of curves this fixed basis is of special importance. The technique will be described presently.

Apparatus¹ required:

- (1) A good colorimeter, preferably the Kober pattern.
- (2) A torsion balance.
- (3) Special platinum capsule for collection of blood.
- (4) Small pair of cross-action forceps, with smooth gripping surfaces, for holding capsule (see Fig. 1).
- (5) Several test-tubes (approximately 2.5 × 8.5 cm.) for dilution of blood and precipitation of its proteins.

¹ The glass-discs are supplied by Messrs Baird and Tatlock (London), Cross Street, Hatton Garden, E.C.; the remainder of the apparatus by Messrs Gallenkamp & Co., 19, Sun Street, E.C. 2

(6) Whatman starch-free filter papers, No. 30 (7 cm. diameter).

(7) Special resistance-glass boiling-tubes, with 7 cc. bulbs and graduated for 12.5 cc. (of same pattern as the Folin and Wu and Mackenzie Wallis tubes; these, however, have only 4 cc. bulbs).

(8) Pipettes of the following capacities: two 1 cc. pipettes graduated in 1/50ths for the 10 % sodium tungstate and $\frac{2}{3} N$ H_2SO_4 solutions; two 2 cc. ordinary bulb-pipettes for the copper and phospho-molybdic acid solutions; one 5 cc. pipette for tungstic acid filtrate.

(9) Burette of 25 cc. capacity for distilled water.

(10) Water-bath (fairly large).

(11) A pair of special coloured glass discs, namely "7.5B," referred to below.

(12) In addition to 10 % sodium tungstate and $\frac{2}{3} N$ H_2SO_4 solutions the following (very carefully prepared) are required:

(a) *Copper solution.* Dissolve 40 g. pure anhydrous sodium carbonate in about 500 cc. distilled water in a litre cylinder. Then add 7.5 g. tartaric acid and when the latter is dissolved and effervescence has ceased, add 4.5 g. crystallised copper sulphate; dissolve without the aid of heat and make up to 1 litre with distilled water. Keep in a dark-coloured bottle. Impurities in the carbonate may give rise to a sediment in the course of a few weeks. If this happens, transfer the clear solution to another bottle.

(b) *Phospho-molybdic acid solution.* 35 g. pure molybdic acid are dissolved in 200 cc. of a 10 % solution of sodium hydroxide and 200 cc. of water added. The whole is now boiled for 20–40 minutes or longer until all traces of ammonia have been driven off, as shown by litmus paper held in the vapour. Cool and dilute to about 350 cc. with distilled water and then add 125 cc. of phosphoric acid (85 % strength) and water to 500 cc.; 2 cc. of this solution when added to 2 cc. of the copper solution should produce complete decolorisation.

(c) *Standard sugar solution.* When about to determine the sugar value of the coloured glasses, the standard sugar solution is required, made as follows. Dissolve 4 g. of pure powdered glucose in about 500 cc. distilled water and make up to 1 litre (a few drops of toluene will preserve the solution for some time, if necessary); 10 cc. of this made up to 1 litre, is the "standard sugar solution."

Preparation of the blue colour from the standard sugar solution and the matching thereof with the coloured glasses (thus determining the colorimeter-scale values of the latter in terms of the former).

Of the freshly-prepared standard sugar solution 5 cc. (containing 0.2 mgm. glucose) are placed in one of the special boiling tubes and 2 cc. of the copper solution added (the solution now reaching the constricted part of the tube). The tube is shaken so as to mix thoroughly the contents and then placed in a vigorously boiling water-bath for *exactly* six minutes. The volume of water

should be fairly large; no lowering of temperature should be noticed when the tube is introduced. On removal of the latter from the bath, 2 cc. of phospho-molybdic acid solution are immediately added, a clear deep-blue solution resulting. Distilled water is added up to the 12.5 cc. mark and the contents mixed by inverting the tube. This is the standard blue solution, which is to be matched with coloured glass.

For this purpose, a long series of pure blue glass slides¹ (5.1×1.8 cm.) was obtained. The 155 slides of the series are accurately graded (.01 to 20.0) as regards depth of colour, and the consecutive colours are so nearly alike that differences can scarcely be detected. It was at once discovered that the colour of the standard solution was not that of pure blue. Investigation proved it to be a mixture of blue and yellow. Accordingly a series of pure yellow glasses, similar to that of the blue, was also employed. After a number of trial experiments, it was found that the blue of the standard solution could be matched exactly by combining one of the blue glasses with a comparatively weak yellow glass. At first it was thought that more accurate estimations of sugar could be obtained by having a number of combinations of blue and yellow glasses, covering the range of colour-intensity derived from blood filtrates, and making use of the approximate combination. Blue glasses of appropriate depths of colour were therefore selected and the necessary correction made with yellow. The latter was determined by placing the standard blue solution (ready for use five minutes after dilution) in one cup of the colorimeter, putting one of the blue glasses underneath the other cup and then adding successively various yellow glasses, until an exact match was effected. At the same time, the cup containing the solution was manipulated so that the colours were also equal in depth. In matching any solution with the fixed depth of colour of the glasses, it is advisable to maintain the cup of the latter at roughly the same level as the cup containing the former. The reading of the cup containing the standard blue solution gives the value of the particular combination of glasses for that solution. After a few trials one gets accustomed to the process and an accurate result is easily obtained. The following are a few of the combinations of glasses with their respective values, as determined by the writer:

No. of glass			Kober colorimeter reading
Blue	+	Yellow	
15.0	+	1.5	= 29.4
12.5	+	1.2	= 24.3
10.0	+	1.0	= 19.4
7.5	+	0.8	= 15.4

No definite relationship necessarily exists between the grading of the glasses and their colorimeter values.

On employing the above four combinations for each estimation of the sugar-content of numerous blood filtrates and glucose solutions of various strengths, it was found that the same result was obtained with each com-

¹ Lovibond Tintometer Glasses (Tintometer Ltd., Salisbury).

bination of glasses. Therefore only one pair of glasses is required. The 15.0 blue + 1.5 yellow, and 12.5 blue + 1.2 yellow combinations are too deep in colour for low concentrations of sugar and so are unsuitable by themselves. In a few instances even the 10.0 blue + 1.0 yellow may be too dark for the purpose; accordingly as a single combination, the use of the 7.5 blue + 0.8 yellow glasses is advised.

Occasionally when only a very light blue solution is developed from the blood filtrate (e.g. through inability to obtain more than minute quantities of blood, as may possibly occur with infants or markedly debilitated persons, etc.), it will be found helpful to use a pair of discs of lighter colour. "5B" (5.0 blue + 0.5 yellow), having a value of 11.4, can be obtained to meet this contingency.

For ease of manipulation the glasses can now be obtained as pairs of discs; these can be conveniently dropped into one of the cups of the colorimeter. In all cases the values of the glasses should be determined individually in order to allow for the personal equation and for any possible slight variation in the composition of the solutions. Again, it is perhaps advisable to check the value of the discs whenever a fresh stock of copper solution is prepared, as slight variations may possibly occur.

On occasions artificial light may be required for the colorimeter. In this connection a 100 watt Osram daylight lamp¹ (blue) has proved most satisfactory.

*Estimation of sugar in blood filtrates by means of the
standard glass discs.*

One can conveniently pipette off 5 cc. of blood filtrate. This is placed in one of the boiling-tubes and treated in the same manner as the standard sugar solution, *i.e.* 2 cc. copper solution are added and thoroughly mixed; placed for *exactly* six minutes in a boiling water-bath; on removal from bath 2 cc. phospho-molybdic acid solution added immediately; solution made up to 12.5 cc. mark with distilled water and, after the lapse of five minutes, compared in the colorimeter with the glass discs. With very little practice, accurate readings are easily obtained.

Calculation. *E.g.* A sample of blood collected from a normal person, 3½ hours after a light breakfast.

Weight of platinum capsule	= 171 mgm.
" " + blood	= 395 "
Weight of blood	= 224 "

The blood was mixed with 7.3 cc. distilled water and 0.3 cc. sodium tungstate solution + 0.3 cc. $\frac{2}{3}$ N H₂SO₄ added (= 8 cc., allowing 0.1 cc. for blood, etc. as above). Of the filtrate 5 cc. were used; this represents $\frac{5}{8} \times 224 = 140$ mgm. blood. The blue solution resulting from this filtrate was

¹ Obtainable from G. E. Co., Kingsway, London

found to match the combination of 7.5 blue + 0.8 yellow discs at the colorimeter reading of 25.9. This pair of discs has a value of 15.4, *i.e.* the standard blue solution if set at 15.4 would effect the same match. Now the value of this pair (or of any of the other pairs) of discs represents 0.2 mgm. glucose (the amount contained in the 5 cc. of standard glucose solution employed).

Hence the amount of sugar contained in the 5 cc. of tungstic acid filtrate, *i.e.* in 140 mgm. blood, = $\frac{0.2 \times 15.4}{25.9} = 0.1189$ mgm.

This is, however, only an apparent value. A correction depending on the variations of copper reduction, must be made before the true value is obtained. The amended value is 0.139 mgm. (derived from the curve I).

Hence % sugar in the blood = $\frac{0.139 \times 100}{140} = 0.099$, which is a normal "fasting level."

The correction of apparent values is a matter of importance; it will now be considered in detail.

Curve of correction for copper reduction.

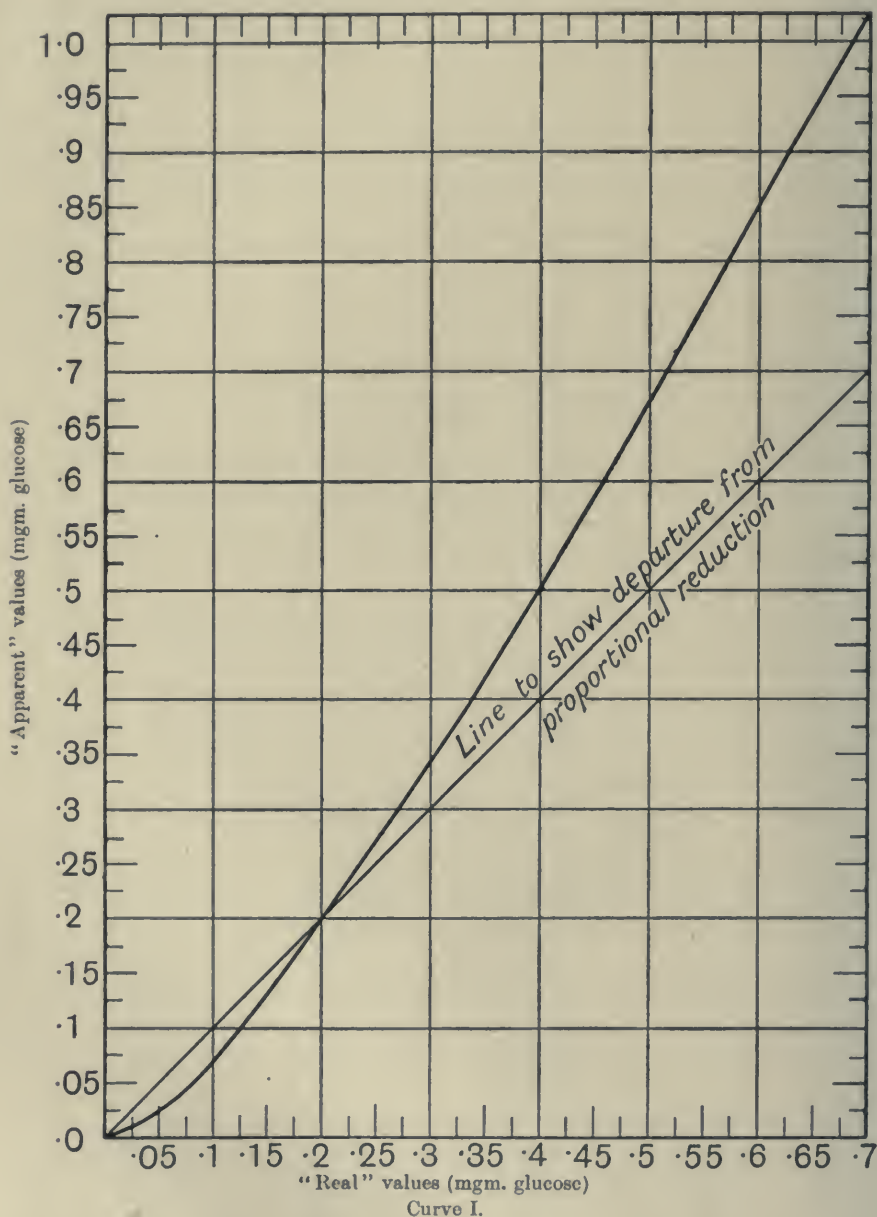
Copper is not reduced proportionately to the amount of sugar present. Therefore cuprous oxide values of solutions of higher or lower sugar concentration than the standard sugar solution do not give accurate values for sugar-content, unless corrected. The writer has worked out a curve of correction for this purpose.

Construction of the curve. As the sugar-content of the blood is estimated from the amount of sugar in 5 cc. of the tungstic acid filtrate, it was decided to employ a series of 5 cc. solutions containing known amounts of pure glucose. One then endeavoured to recover experimentally the sugar content of each of these solutions in exactly the same way as for a blood filtrate. The experi-

Table showing "real" and "apparent" values.

(Mgm. glucose in 5 cc. solution from which the curves are plotted.)			
"Real value" (mgm. glucose)	"Apparent value" (mgm. glucose)	"Real value" (mgm. glucose)	"Apparent value" (mgm. glucose)
0.050	0.025	0.525	0.720
0.075	0.045	0.550	0.763
0.100	0.070	0.575	0.808
0.125	0.100	0.600	0.850
0.150	0.133	0.650	0.938
0.175	0.165	0.700	1.025
0.200	0.200	0.800	1.220
0.225	0.233	0.900	1.392
0.250	0.266	1.000	1.567
0.275	0.305	1.100	1.750
0.300	0.343	1.200	1.900
0.325	0.379	1.300	2.008
0.350	0.420	1.400	2.070
0.375	0.460	1.500	2.115
0.400	0.504	1.600	2.140
0.425	0.545	1.700	2.160
0.450	0.587	2.000	2.160
0.475	0.630	2.400	2.160
0.500	0.675	3.000	2.160

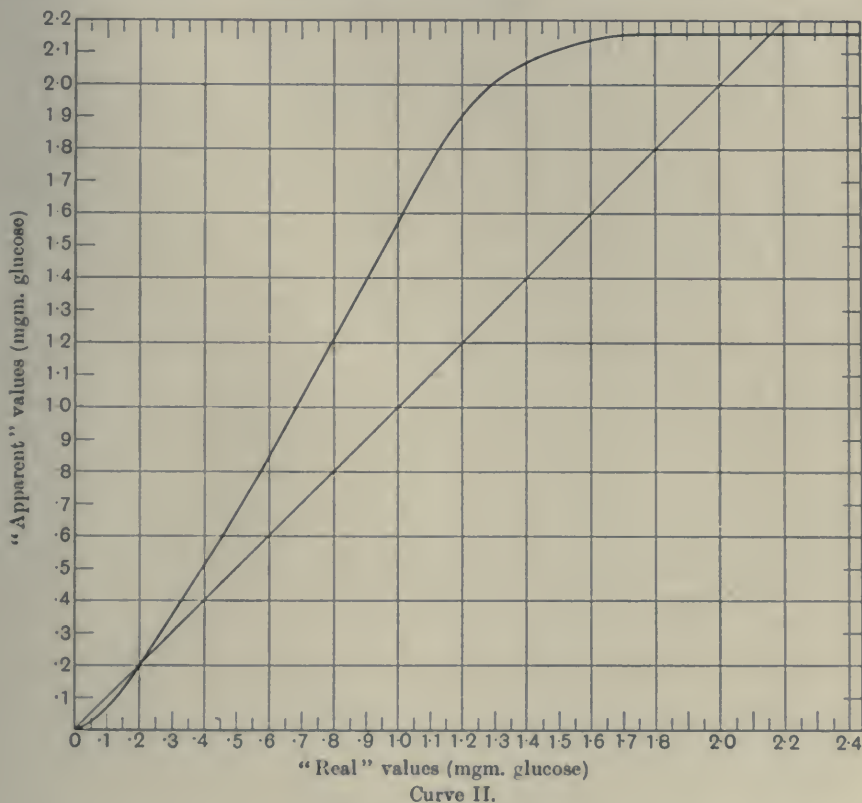
mental result is called the "apparent value" in contrast to the "real value"; these have been plotted one against the other. Two curves are given (curves I and II). Curve I is the one intended for use in this method; from the values



tabulated, it can, with advantage, be drawn to a much larger scale. Curve II is curve I continued by making use of the remaining values; the scale is much smaller.

In preparing the "real value" solutions, great care was taken to avoid error. The experiments were conducted on freshly-prepared solutions; six was found to be a convenient number with which to deal at one time.

First of all, 4 g. of pure powdered glucose was dissolved in about 500 cc. distilled water and the volume made up to 1 litre. If 10 cc. (containing 0.04 g. glucose) of this solution (X) be diluted to 1 litre, then 5 cc. of the resulting solution (Y) will contain 0.2 mgm. glucose. Solution (Y) is really



the "standard sugar solution" already mentioned. By varying the number of cc. of solution (X) various amounts of glucose are obtained in the 5 cc. solutions. Thus, 5, 10, 15, 20, 25, 30, etc., cc. of solution (X) when made up to 1 litre produced solutions, 5 cc. of which contained 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, etc., mgm. glucose respectively. A value like 0.275 mgm. was obtained by preparing solutions containing 0.250 and 0.300 mgm. glucose (*i.e.* 12.5 cc. and 15 cc. solutions (X) used) in 5 cc. and mixing, say, 20 cc. of each; 5 cc. of the mixture contained 0.275 mgm. glucose. The "apparent values" were determined by placing 5 cc. of "real" or known value solutions in boiling-tubes, adding 2 cc. of the copper solution and proceeding as in the case of blood filtrates to make the colorimetric estimations.

An example will make this clear, and, at the same time, demonstrate the equivalence of the several pairs of discs previously referred to, thus illustrating the principle on which the method of using coloured glass standards is founded. In actual practice only the 7·5B pair would be used. (The 7·5 blue + 0·8 yellow combination of discs is conveniently termed 7·5B; similarly one speaks of 10B, 12·5B and 15B.) To determine the "apparent" value of a 5 cc. solution containing 0·30 mgm. glucose, the blue-coloured solution is developed in the usual way and placed in one cup of the colorimeter and compared with a pair of discs placed in the other cup.

The following were the colorimeter readings obtained for the discs:

$$\begin{array}{ll} 7\cdot5B = 9\cdot0, & 12\cdot5B = 14\cdot1, \\ 10B = 11\cdot3, & 15B = 17\cdot1. \end{array}$$

Now the values of 7·5B, 10B, 12·5B and 15B are 15·4, 19·4, 24·3 and 29·4 respectively, each value representing 0·2 mgm. glucose. Therefore the "apparent" values given by

$$\begin{aligned} 7\cdot5B &= \frac{.2 \times 15\cdot4}{9\cdot0} = 0\cdot342 \text{ mgm. glucose.} \\ 10B &= \frac{.2 \times 19\cdot4}{11\cdot3} = 0\cdot343 \quad , \quad , \\ 12\cdot5B &= \frac{.2 \times 24\cdot3}{14\cdot1} = 0\cdot344 \quad , \quad , \\ 15B &= \frac{.2 \times 29\cdot4}{17\cdot1} = 0\cdot343 \quad , \quad , \end{aligned}$$

Mean value = 0·343 mgm. (see table).

As regards the complete curve—curve II—it will be noticed that at three points the "real" and "apparent" values coincide:

at zero,
at 0·2 mgm.,
at 2·16 mgm. ("real" value).

The values are equal at 0·2 mgm. because this is the amount of glucose in 5 cc. of the standard sugar solution, from which the disc-values have been determined. The values are the same at 2·16 mgm. because all the copper in the 2 cc. of stock solution (theoretically amounting to 2·29 mgm. Cu) has been reduced by a 5 cc. solution containing 1·7 mgm. glucose; the "apparent" value at this point is 2·16 mgm., and increase of the glucose used for reduction does not alter it.

Numerous experiments have been carried out which prove the reliability of the curve in enabling one to recover experimentally the percentage of sugar from solutions of known strength. *E.g.* To recover the percentage of glucose in a solution of 0·25 % strength: The platinum capsule was filled with this solution and weighed on the torsion balance, 231 mgm. of the solution being taken.

Capsule + solution were then put into 7·8 cc. of distilled water. 5 cc. of the mixture was placed in a boiling-tube, 2 cc. copper solution added, and the process of estimating the sugar-content continued as already described.

Colorimeter readings obtained:

$$7.5B \text{ (value } 15.4) = 7.0. \therefore \text{ "apparent" value} = \frac{0.2 \times 15.4}{7.0} = 0.440 \text{ mgm.}$$

$$10B \text{ (value } 19.4) = 8.8. \therefore \text{ "apparent" value} = \frac{0.2 \times 19.4}{8.8} = 0.441 \text{ mgm.}$$

$$\therefore \text{ Mean "apparent" value} = 0.4405 \text{ mgm.}$$

If this be left uncorrected the percentage of sugar obtained for the 0.25 % solution would be:

$$\frac{0.4405}{\frac{5}{100}(231)} \times 100 = \frac{44.05}{144.4} = 0.305 \%$$

(where $\frac{5}{100} \times 231$ or 144.4 = mgm. glucose solution used).

But on making the correction from the curve, the "apparent" value 0.4405 mgm. becomes the "real" value 0.362 mgm., and therefore the amount of sugar found in the 0.25 % solution = $\frac{0.362 \times 100}{144.4} = 0.2507 \%$.

Again, the method has been thoroughly tested by recovering added glucose from blood:

For this purpose two capsules were required. Two simultaneous samples of blood were taken, No. 1, 230 mgm.; No. 2, 214 mgm.

On being weighed, No. 1 blood was put immediately into 7.3 cc. distilled water and mixed. No. 2 blood was mixed in 7.1 cc. distilled water. The proteins of No. 1 were precipitated in the usual way and the solution filtered. The capsule now available was employed to weigh out a quantity of 0.25 % glucose solution, 182 mgm. of which were taken.

This was added to No. 2 blood solution. The addition of the precipitants made the volume up to 8 cc.

5 cc. of each filtrate was taken and the sugar content estimated:

(a) *Blood alone:*

$$\text{Amount used} = \frac{5}{100} \times 230 = 143.75 \text{ mgm. blood.}$$

Using 7.5B the reading was 18.3.

$$\therefore \text{ "apparent" amount sugar in } 143.75 \text{ mgm. blood} = \frac{0.2 \times 15.4}{18.3} = 0.1683 \text{ mgm.}$$

$$= 0.1780 \text{ mgm. (corrected from curve I).}$$

$$\therefore \text{ sugar in the blood} = 0.1238 \%$$

(b) *Blood + Glucose:*

Amount blood used = $\frac{5}{100} \times 214 = 133.75$ mgm. and this contains (using above %)

$$\frac{133.75 \times 0.1238}{100} = 0.1656 \text{ mgm. glucose.}$$

The 7.5B reading for the blood + glucose = 5.2.

$$\therefore \text{ "apparent" amount glucose in the mixture} = \frac{0.2 \times 15.4}{5.2} = 0.592 \text{ mgm.}$$

$$= 0.452 \text{ mgm. (corrected).}$$

Now amount glucose solution used = $\frac{5}{100} \times 182 = 113.75$ mgm.

This amount therefore contains $0.4520 - 0.1656 = 0.2864$ mgm. glucose.

$$\therefore \text{ amount of glucose in the } 0.25 \% \text{ solution} = \frac{0.2864 \times 100}{113.75} = 2.519 \%$$

In cases of hyperglycaemia the importance of the curve correction is still more noticeable than with normal blood.

With regard to the solutions used in the method it may be pointed out that the copper solution should be made up freshly every four months; the phospho-molybdic acid, $\frac{2}{3}$ *N* H_2SO_4 and 10 % sodium tungstate solutions seem to keep indefinitely. Attention is directed to the quality of the sodium tungstate used. Carbonates are usually present in commercial tungstates. According to Folin and Wu, these should not exceed a certain amount. On titrating 10 cc. of the 10 % sodium tungstate with *N*/10 HCl , using one drop of phenolphthalein as indicator, not more than 0.4 cc. of the acid should be required. The amount of $\frac{2}{3}$ *N* H_2SO_4 used in the precipitation process has been so regulated as to conform to this standard. No difficulty, however, will be experienced if care be taken to obtain tungstate of good quality. The very slight variations that may occur in the tint of the blue colour developed from blood filtrates are sometimes attributable to impurities in the tungstate; nevertheless an accurate colorimeter reading is easily made.

Again, it should be noted that when the blood is properly coagulated by the tungstic acid, the coagulum changes gradually from pink to chocolate-brown. If this change does not occur within a few minutes, the coagulation is incomplete. In such an emergency an extra drop of $\frac{2}{3}$ *N* H_2SO_4 will usually produce the desired effect.

As regards the care of the platinum capsule: it is shaken up in a couple of changes of water in the test-tube in which it remained on filtering the blood precipitate; then it is dipped successively in absolute alcohol and ether; after one passage through a Bunsen flame it is ready for use again.

SUMMARY.

A rapid and reliable method of estimating sugar in the blood has been described. The number of manipulations has been reduced to a minimum, thus making it particularly valuable in routine clinical work.

The following are its main features:

(1) Collection and weighing of blood in a special platinum capsule—simple and accurate:

(a) The capsule remains constant in weight; numerous weighings can thus be avoided.

(b) In the process of dilution all the blood is immediately diffused, therefore "sugar-extraction" is unnecessary.

(2) Accuracy is gained by making the fullest use of the blood available.

(3) A fixed colour standard in glass. The selection of the standard and the determination of its value are described. The use of the standard (issued as a pair of glass discs—"7.5B") saves much time and labour. Further, it pro-

vides an unchanging basis for the estimations; this is of importance in the construction of blood-sugar curves.

(4) Use of a curve of correction for copper reduction; an appreciable, if not considerable, error in sugar estimation is thereby eliminated.

(5) The technique is so simple that one can carry out concurrently the estimations necessary for several blood-sugar curves.

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XVII. THE SYNTHESIS OF IMINAZOLYLGLYCINE, THE LOWER HOMOLOGUE OF HISTIDINE.

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(Received January 16th, 1923.)

ENGELAND [1908] isolated from human urine a small quantity of a picrolonate, melting at 244° , to which he assigned the formula $C_5H_7O_2N_3$, $C_{10}H_8O_5N_4$. From this, by decomposition with hydrochloric acid, he obtained a solution, which gave a red coloration with sodium diazobenzenesulphonate. He concluded from these observations that the substance was the lower homologue of histidine.

It was believed that the synthesis of this amino-acid would be useful, partly as it might to some extent support Engeland's conclusion as to the identity of his substance, and partly because a knowledge of its properties might facilitate its isolation from other natural sources.

The only method available for the synthesis appeared to be that of Strecker in which, by the joint action of potassium cyanide and ammonium chloride, an aldehyde is converted into an amino-nitrile, from which the amino-acid is obtained by hydrolysis. The iminazolyl-4 (or 5)-formaldehyde [Pyman, 1916] was prepared by the oxidation of iminazolyl-4 (or 5)-methyl alcohol, and the latter substance was obtained from citric acid by the method used by Pyman [1911] in his fundamental syntheses of histamine and histidine. The additional experimental details supplied by Koessler and Hanke [1918] were useful in securing good yields in the various stages of this synthesis.

The iminazolylglycine was obtained impure in rather small yield and had to be purified through the picrolonate. The highest melting point of this synthetic salt, 243° (uncorr.) is in close agreement with Engeland's value of 244° . So far as this slender evidence goes, it tells in favour of Engeland's view as to the nature of the substance which he isolated from urine. As was to be expected, iminazolylglycine closely resembles histidine; it does not, however, give Knoop's reaction.

EXPERIMENTAL PART.

Preparation of iminazolyl-4 (or 5)-glycine.

Equimolecular proportions of iminazolyl-formaldehyde (1 g.), potassium cyanide (0.65 g.) and ammonium chloride (0.53 g.) were dissolved in the minimum of water (5 cc.) and allowed to stand at room temperature for 48 hours. The solution gradually darkened in colour and after a few hours became almost opaque. Attempts were made to prevent this by filling the

reaction vessel with nitrogen and hydrogen, but were unsuccessful. It is important that the concentration of the reacting substances be kept as high as possible; on one occasion, when about twice the usual volume of water was used, scarcely a trace of amino-acid was obtained.

The amino-nitrile was not isolated, but was straightway hydrolysed by adding to the reaction mixture an equal volume of concentrated hydrochloric acid and heating on the boiling water-bath for three hours. The liquid was then evaporated to dryness and the residue dissolved in water. Chlorides were precipitated by silver nitrate, in the presence of nitric acid. After filtration, silver nitrate was added in sufficient quantity to give a brown precipitate with barium hydroxide, and the silver compound of the amino-acid, mixed with silver oxide, was filtered off, washed, and decomposed by hydrogen sulphide. The filtrate from the silver sulphide was boiled to expel hydrogen sulphide, freed from barium by sulphuric acid, and evaporated. The resulting crude amino-acid was recrystallised from dilute alcohol. After several recrystallisations it formed sheaves of microscopic colourless plates, which darkened at 220° and melted with decomposition at 254°. It gave Pauly's reaction for glyoxalines, the triketohydrindene reaction for amino-acids, but not Knoop's test for histidine, neither in its original form, nor as modified by Hunter [1922]. It was readily soluble in water, slightly in methyl, almost insoluble in ethyl alcohol.

Nitrogen by micro-Kjeldahl.

4.92 mg. (dried at 120°) gave 1.275 mg. N N = 25.9 %.

Calculated for $C_5H_7N_3O_2$ N = 26.4 %.

The yield was variable and somewhat low: from 1 g. of the aldehyde, 0.25 to 0.50 g. of crude amino-acid was obtained. The *phosphotungstate* crystallises from hot water in glistening rhombic plates.

Preparation of the picrolonate.

The *monopicrolonate* was prepared by adding a hot saturated aqueous solution of rather less than the theoretical quantity of picrolonic acid to a concentrated aqueous solution of the amino-acid [Levene and Van Slyke, 1912]. On cooling, the picrolonate separated in deep yellow crystals which, after recrystallisation from water and from ethyl alcohol, melted and decomposed at 243°—a figure in good agreement with that given by Engeland [1908].

Nitrogen by micro-Dumas.

4.14 mg. (dried at 100°) gave 0.854 cc. N_2 at 11° and 762 mm. N = 24.66 %.

Calculated for $C_5H_7N_3O_2 \cdot C_{10}H_8N_4O_5$ N = 24.2 %

Estimation of picrolonic acid.

Picrolonates are decomposed by nitron, and as in the case of nitrates and picrates, the nitron compound is practically insoluble in water. The reaction can therefore be used for the estimation of picrolonic acid, and gives satisfactory results even on the micro scale.

Thus 21.09 mg. pure picrolonic acid gave 45.92 mg. nitron picrolonate.

% picrolonic acid = 99.80.

12.16 mg. 4- (or 5)-iminazolyglycine picrolonate gave 17.43 mg. nitron picrolonate.

% picrolonic acid = 65.7.

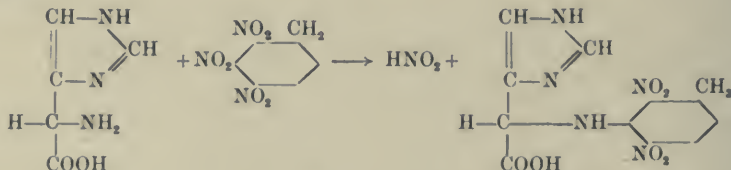
Calculated for $C_5H_7N_3O_2 \cdot C_{10}H_8N_4O_5$ 65.2 %.

Decomposition of the picrolonate.

The picrolonate was dissolved in dilute hydrochloric acid and the free picrolonic acid thus formed was removed by extraction with benzene and finally with ether. The residual solution of the amino-acid hydrochloride was evaporated on the water-bath and the residue was recrystallised from dilute alcohol. It was, however, hygroscopic, and decomposed when attempts were made to dry it at 100°. Similar results were obtained when the hydrochloride was prepared by dissolving the amino-acid in hydrochloric acid.

C. 4- (or 5)-iminazoly-N . 2.4-dinitrotolyl-3-glycine.

β - and γ -trinitrotoluene condense with compounds containing a primary amine group, with elimination of nitrous acid [Barger and Tutin, 1918]. In this case, 2 . 3 . 4-trinitrotoluene was used, the 3-nitro-group disappearing in the process.



20 mg. of the amino-acid and 60 mg. of 2.3.4-trinitrotoluene (a considerable excess) were dissolved in dilute alcohol and heated under a reflux condenser for three hours. The liquid gradually darkened to a deep yellow. It was evaporated to dryness, and the unchanged trinitrotoluene removed by repeated extraction with hot benzene. The deep yellow crystalline condensation product remained behind. It was very soluble in water, but insoluble in ethyl alcohol. Recrystallised from dilute alcohol, it decomposed violently at 270°.

The nitrogen content was estimated by a modified micro-Kjeldahl. Eckert [1913] was able to use the Kjeldahl method in presence of aromatic nitro-groups by adding sulphur as a reducing agent. 0.2-0.5 g. of substance was mixed with 0.4 g. of sulphur and heated on the water-bath for one hour with 20 cc. of 30-40 % fuming sulphuric acid. The further procedure was as usual.

In adapting this method for use on the micro-scale, it was found advisable to increase the amount of sulphur to two or three times the weight of substance, and also to heat on the water-bath for two hours. Shorter heating did not give satisfactory results with the substances tried, which, however, as they contained two nitro-groups, afforded a fairly severe test of the method.

6.95 mg. of *N*. 2.4-dinitrotolyl-3-histidine were found to contain 1.407 mg. nitrogen.

$$N = 20.3 \%$$

Calculated for $C_{13}H_{13}N_5O_6$ $N = 20.9 \%$.

3.58 mg. of *C*. 4- (or 5)-iminazolyl-*N*. 2.4-dinitrotolyl-3-glycine contained 0.7752 mg. nitrogen.

$$N = 21.65 \%$$

Calculated for $C_{12}H_{11}N_5O_6$ $N = 21.8 \%$.

I wish to express my sincere thanks to Professor Barger for suggesting this research, and for much valuable assistance.

The expenses of this work were partly defrayed by a grant from the Moray Research Fund of this University, for which my thanks are also due.

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XVIII. THE FORMATION OF VITAMIN A IN PLANT TISSUES. II.

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(Part of a Thesis approved for the degree of D.Sc., University of London.)

(*Received November 18th, 1922.*)

INTRODUCTION.

INVESTIGATORS who have used plant tissues as a source of vitamin A in research on its influence on metabolism and growth of animals have frequently observed that the green leaf is richer in this factor than the seed of the plant; and in those plants which form "hearts" such as the cabbage and lettuce, they have found that the green outer leaves are richer than the more or less etiolated inner ones [McCollum, Simmonds and Pitz, 1917; Steenbock and Gross, 1919; Hume, 1921]. No one made any exact comparisons between these two types of tissue until Coward and Drummond [1921] examined etiolated and green shoots of various plants, using very small doses for the feeding tests. They found that shoots which had been exposed to light were very much richer in vitamin A than shoots of the same species grown in darkness; in fact the etiolated shoots in most cases were entirely inactive in the doses used in the tests. In one test of the unsaponifiable matter (shown by Steenbock, Sell and Bluell [1921] to contain the vitamin A if present) from the respective shoots, the dosage must have corresponded to a very great many fresh shoots, and as no growth was obtained from the fraction from the etiolated shoots, it would appear to be possible that sunlight had not only increased the concentration of the vitamin in the green shoot but had, perhaps, even initiated its formation in the plant. This suggested a possible association of the vitamin formation with the photosynthetic activity (carbon assimilation) of the plant and a further series of experiments was planned to determine other conditions of its formation. In the meantime, however, a paper appeared by Wilson [1922] in which he contended that "photosynthesis is not necessary for the production of vitamin A in plants." Hence it seemed desirable to repeat one or two of the former experiments, using the same plant that Wilson had used in his work.

METHOD FOR TESTING A TISSUE FOR VITAMIN A.

The general method of procedure was that described by Drummond and Coward [1920], with the modification described later by Coward and Drummond [1921]. The tissue to be tested was cut from the plant, transferred to a box and carried at once to the animal house where it was put inside the nesting box of the cage. As it was given to the rat early in the morning before the daily ration of basal diet, it was practically always eaten at once and so was not subjected to further influence of light than the experiment demanded. Where the fresh tissue appeared to be unpalatable or poisonous, large masses of it were saponified and the unsaponifiable matter was extracted with ether in the usual way, care being taken to prevent oxidation by carrying out the whole process as far as possible in an atmosphere of carbon dioxide. At each stage of the extraction the separating funnels were filled with carbon dioxide, and during filtration a current of the gas was passed over the filter paper the whole time. The unsaponifiable matter was given directly to the rats by dropping from a glass rod into their mouths and thus the dosage was easily determined. Where seedlings of a definite age were to be fed, seeds were set in plant pots each day and the series used daily when the required age had been attained by those first set.

I. THE INFLUENCE OF SUNLIGHT.

Wilson drew the conclusion quoted above from the fact that he obtained just as good growth by including 5 % of dried etiolated wheat shoots in his basal ration as by including 5 % of dried green shoots. Apparently this experiment was performed on rats which had not previously become steady in weight on a diet deficient in vitamin A as no preparatory period is mentioned and the experiment was started when the rats' weights ranged from 40 to 60 g. each. It is becoming a matter of common experience that rats whose mothers have received large supplies of vitamin A during pregnancy and lactation, can grow from this weight to maturity on diets containing only minimal traces of vitamin A. Moreover, Wilson's basal mixture contained 20 % of lard, and Daniels and Loughlin [1920] showed that lard could give growth; and Drummond, Zilva, Golding and Coward [1920] have shown very definitely that lard from pigs whose diet has contained vitamin A may itself contain the vitamin in large enough quantities to restore normal growth to young rats when administered to them as 10 or 15 % of their diet. The fact that two of Wilson's rats died on this basal diet after 8½ and 9 weeks respectively is scarcely sufficient proof of its total deficiency in vitamin A. In a later experiment, three of Wilson's rats became steady in weight on his basal diet at body weights of 130, 120 and 107 g. respectively. These are so high that they indicate a rich reserve of vitamin at the beginning of the preparatory period or the presence of some small amount of vitamin in the basal diet. Also, a resumption of growth (gained in this experiment by adding 8 % of dried etiolated wheat shoots to the diet) is usually obtained by much

smaller doses of vitamin A in rats of this weight than are necessary for rats which have become steady at a lower weight. These considerations all seem to indicate that the amount of vitamin A in etiolated wheat shoots must be very small indeed and without a test of wheat seeds on rats with a similar basal diet, it seems scarcely possible to draw a comparison between the vitamin content of etiolated shoots and that of the seeds themselves. It is much to be regretted that, while making this investigation, Wilson did not proceed to make a comparison between etiolated and green wheat shoots in smaller dosage, for he says that "all physiological activity must be more intense in the sprouts grown in the light than in those grown in the dark."

In view of this criticism it seemed desirable to repeat the light experiment on wheat shoots. Two series of sowings of wheat seeds were made on successive days for a period of six weeks, one series being placed immediately in a large airy dark room and the other in a verandah in sunlight, protected above and on two sides by glass. Twelve days after the first sowing the respective shoots were given to experimental rats whose weights had become stationary on our basal diet, and each day for a period of three weeks or longer, one shoot only was given to each rat. The average weight of a fresh etiolated shoot was 0.14 g., its dry weight being 0.012 g. The average weight of a fresh green shoot was 0.12 g., its dry weight 0.011 g. In case even such a small amount of etiolated shoot should give growth, a test was made of one ungerminated seed per rat per day, though in view of our previous findings on wheat seeds this appeared superfluous. It did indeed prove to be so—no growth being shown by any of the four rats and three of them declining in weight and dying. Six rats were used for testing the etiolated wheat shoots, fair growth being obtained in one case, slight growth in two and none in the other three. In marked contrast to these results are those obtained with the green shoots: in each of three rats normal growth was resumed (Fig. 1).

Confirmation of this result was obtained with extracts of unsaponifiable matter from etiolated and green shoots respectively, though the amounts of such matter were very small and only sufficed for ten days' feeding in a dosage of 5 mg. daily. Three rats on the fraction from the etiolated shoots lost between them, 11 g. in body weight. The three rats on the fraction from the green shoots gained 21 g. Each dosage was equivalent to 15 fresh shoots though it is impossible to say what loss occurred during the process of extraction.

A similar test was carried out on shoots of the yellow maize and also on shoots of the white maize. For the former of these, yellow maize purchased at an ordinary corn dealer's was used and for the latter, Carter's white Horse-tooth maize was used. The seeds were again set on successive days and when 12 days old used for feeding, but only a small part of the oldest leaf (0.15 g.) was used each day. Striking differences in the growth-promoting powers between the etiolated and green shoots in each case were again found. The etiolated leaf of the white maize was entirely inactive in the quantity fed,

while the same amount of the green shoot produced normal growth; the etiolated shoot of the yellow maize produced growth in one rat, but none in two, while the green shoot produced good growth in three different rats (Fig. 2). Those familiar with the biological test for vitamin A will recognize the irregular behaviour sometimes shown by an individual rat, and hence the necessity of using groups of animals for any one test.

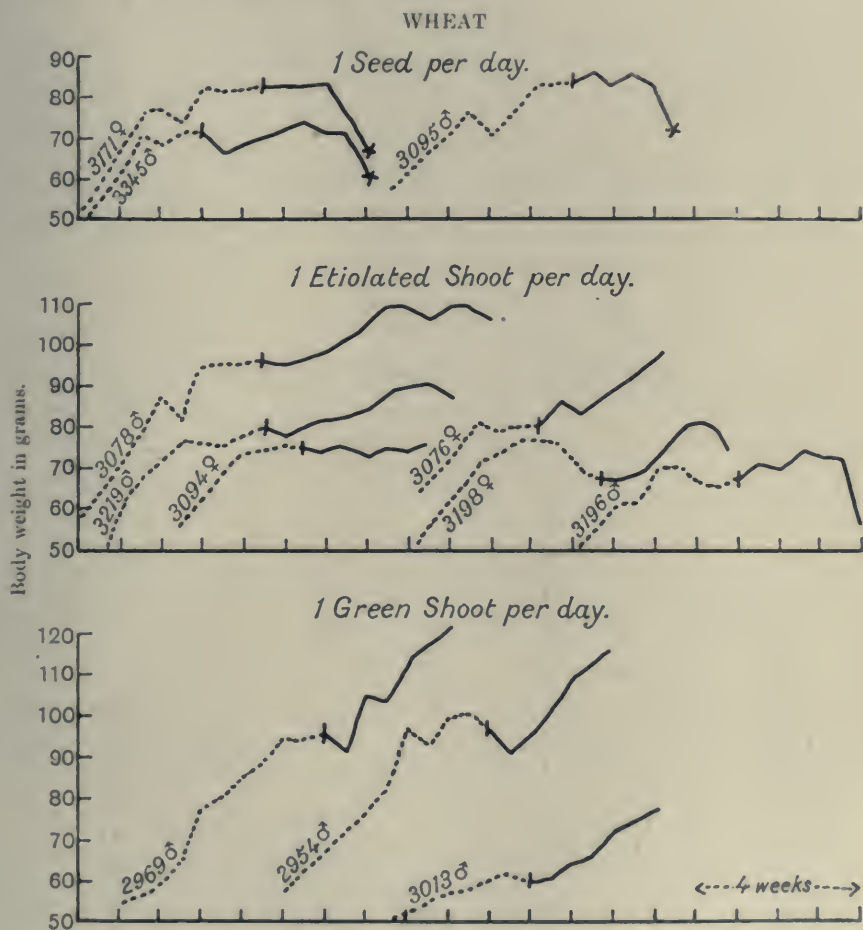


Fig. 1.

Preparatory period on A-deficient diet shown in dotted lines.

Thus, while it would be impossible to deny the existence of very small quantities of vitamin A in etiolated seedlings, it would appear to be established that the amount is increased considerably by exposing the seedlings to sunlight.

It was also thought desirable to examine the influence of electric light on the formation of vitamin A. For this purpose a series of sowings of seeds of *Helianthus annuus* (sunflower) was made. One test was made on the etiolated

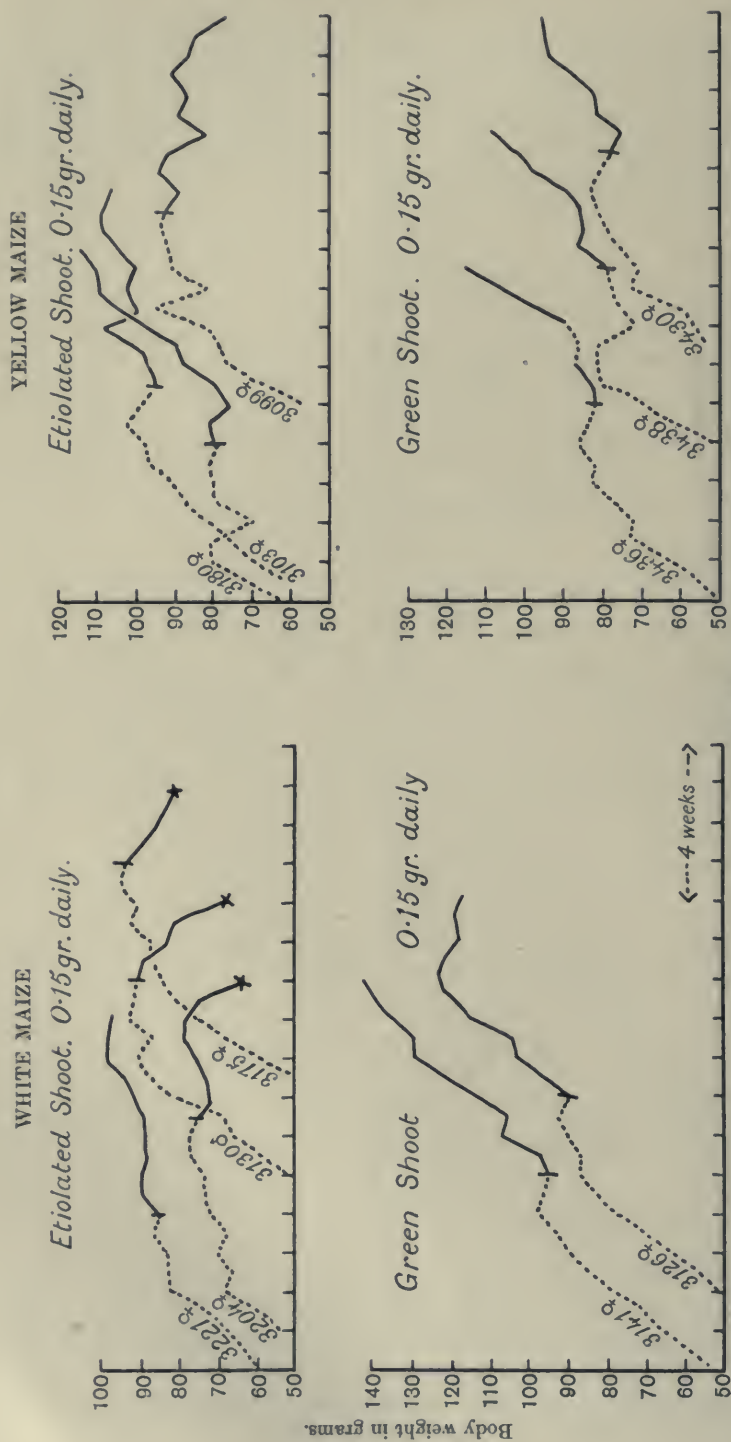


Fig. 2.

Preparatory period on A-deficient diet shown in dotted lines.

seedlings at the age of nine days, a further one on seedlings that had grown in the dark for nine days and then had been subjected to various periods of illumination from an electric light of 32 candle power at a distance of 16 inches. Various trials were made and most marked contrast was obtained with shoots that had been treated for three days with 10 hours' illumination and 14 hours' darkness (when the lights were turned off at night) alternately. The first test with two etiolated seedlings produced only very slight growth, the second test with two illuminated seedlings produced good growth which was only slightly subnormal. A further test on seedlings which had been illuminated as the last set and then kept in the dark for eight days gave, in two rats out of three, growth at least as good as that from the shoots which had been eaten directly after illumination. Hence it would appear that electric light is also effective in increasing the rate of formation of vitamin A, though no comparison of its power relative to sunlight was attempted; and it may also be concluded that the store is not immediately used up by the plant (Table I).

Table I.

	Daily dose	Body-weight in g.					
		0	7	14	21	28 days	
<i>Helianthus annuus</i> :							
Etiolated seedlings, 9 days old	2 seedlings	58	61	68	75	77	Slight
		60	63	73	75	69	
Green seedlings, after 3 days' electric light	2 seedlings	75	88	96	106		Fairly good
		71	76	80	89		
		63	74	78	80		
Green seedlings, after 3 days' electric light followed by 8 days in the dark	2 seedlings	79	96	105	—	—	Good
		86	96	108	—	—	
Green seedlings, 14 days old, after 1 day in electric light	2 seedlings	91	105	100	95	104	Slight
		90	80	91	96	102	
		70	65	75	81	81	
<i>Tradescantia</i> :							
	Fresh Mat.						
Grown in Sachs solution	1.0 g.	95	112	115	119	122	Good
		83	100	116	125	136	
		95	95	107	105	117	
Grown in solution without calcium	1.0 g.	72	84	100	105	104	Fairly good
		67	76	86	90	88	
		66	66	68	70	70	

By a further test of growing etiolated seedlings for 14 days in the dark and then exposing them to 10 hours' electric light and 14 hours' dark again, it was shown that no better growth-promoting powers were developed than had been in the nine days old seedlings similarly treated. Thus in vitamin formation in *Helianthus annuus* there is no increased activity through delay in starting it, as Briggs [1920] found was the case with the photosynthetic activity (carbon assimilation) of seedlings of *Phaseolus* (Table I).

II. INDEPENDENCE OF CARBON DIOXIDE IN THE AIR.

As the formation of vitamin A has hitherto been generally associated with those tissues showing at some time photosynthetic activity (carbon assimilation) it seemed advisable to investigate the various factors in this process to see whether they were also factors in the process of formation of vitamin A.

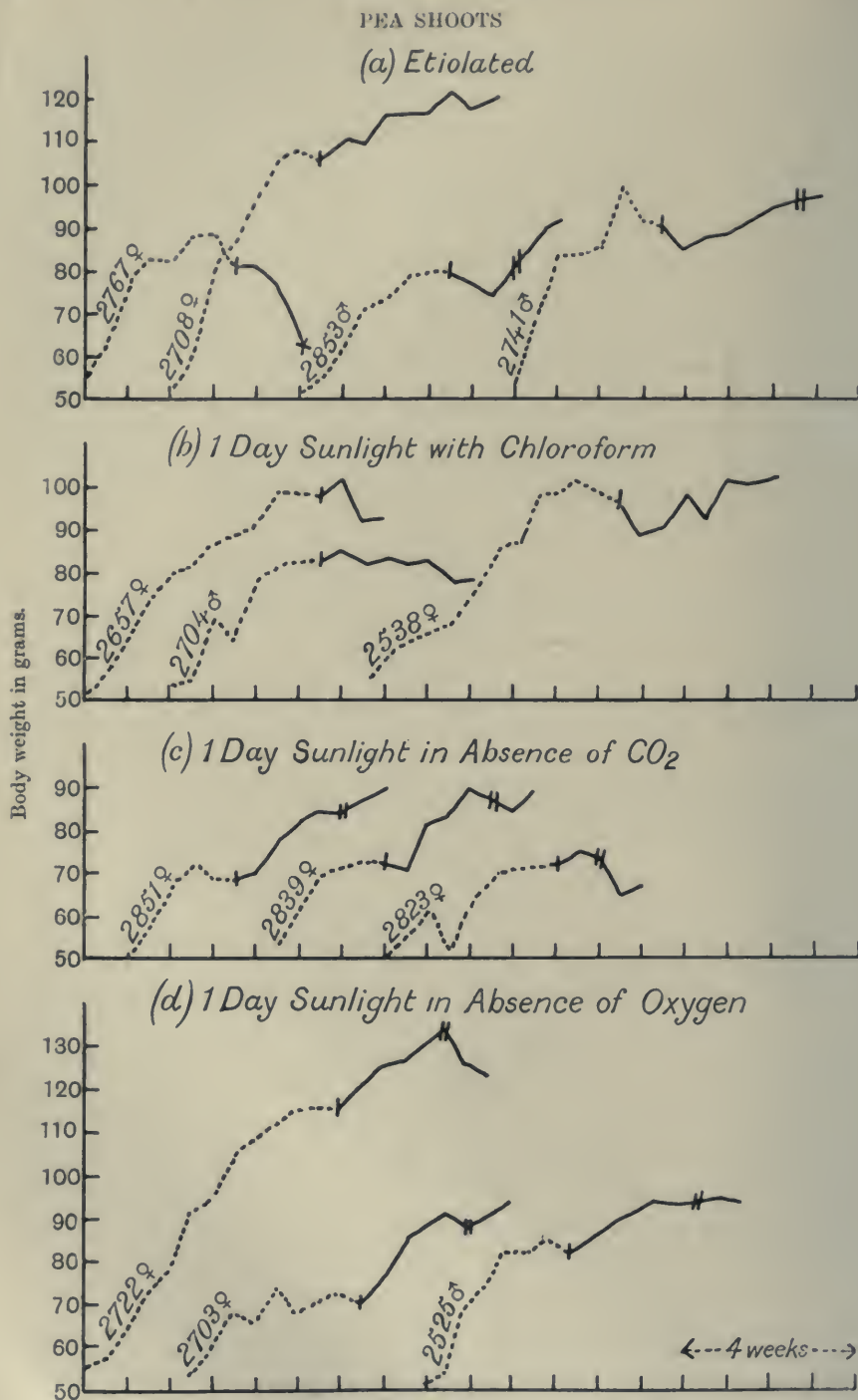


Fig. 3.

Preparatory period on A-deficient diet shown in dotted lines.

The influence of carbon dioxide in the air was first tested. This and the following two tests, together with a control, were run concurrently during May 1922 when, fortunately, there was a succession of sunny days practically without an interruption throughout the experiment. The plan adopted was to cut off shoots of etiolated pea seedlings 12 days old, and place their cut ends in a small tube of fresh tap water in the apparatus each day for 24 hours. The apparatus consisted of a large photographic dish with a layer of mercury, and a bell jar of about 5 litres capacity. Within this stood a wide low dish of 6-inch diameter containing strong potash solution—and the tube containing the cut shoots stood in the middle of this. Thus the shoots would be practically free from carbon dioxide, any that was given out by respiration probably being absorbed by the potash. The whole apparatus stood in a large south window.

A daily supplement of one of these shoots produced growth in rats of the type that is described by Zilva and Miura [1921] as resulting from small doses of vitamin A. That is, the rats grew pretty well for a short time (two weeks), then began to slow down in rate, but on administering two shoots further growth was resumed. One rat only failed to respond to the increased dosage (Fig. 3).

A control of one etiolated pea shoot gave a negative result in three rats. Hence the formation of vitamin A in the plant must be considered as independent of the presence of carbon dioxide in the surrounding atmosphere, if one may ignore the possibility of utilisation of the small quantities admitted to the apparatus in placing the shoot in it, for it might be some time before all the gas became absorbed by the potash (Fig. 3).

III. INDEPENDENCE OF OXYGEN IN THE AIR.

To determine whether oxygen is necessary for the formation of vitamin A in plant tissues, an apparatus was set up similar to that used in the last test but a solution of pyrogallol (10 %) in caustic soda (80 %) was used in place of the potash. Most of the air was blown out each day with CO_2 before the fresh seedlings were put into it, in order to ensure as complete an absorption as possible of the remaining oxygen by the pyrogallate. The apparatus stood in the same window as the last one so that it might receive equal illumination.

The results of the feeding test were very similar to the last, three rats resuming growth for about a fortnight, then becoming stationary. One rat grew again on the addition of two shoots instead of one. Thus it appears that vitamin A can be synthesised by the plant practically in the absence of oxygen in the surrounding atmosphere (Fig. 3).

IV. INFLUENCE OF CHLOROFORM IN THE AIR.

Very small traces of chloroform in the atmosphere are sufficient to inhibit the process of carbon assimilation in a plant. It was determined to find out whether vitamin formation was affected similarly. Cut ends of etiolated pea

shoots 12 days old were placed in a small jar of water for 24 hours under a bell jar in a south window similar to the one used above, and a small flask of chloroform, open, was also placed under it. The base of the bell jar was not sealed with mercury—fresh air was allowed to enter where it could. The shoots remained apparently healthy and developed a decided green tint.

A ration of one shoot per rat per day so treated failed to restore growth in three rats; hence it may be concluded that chloroform inhibits the formation of vitamin A in plant tissues (Fig. 3).

V. INDEPENDENCE OF CHLOROPHYLL.

One difference between the shoots deprived of oxygen in Exp. III and those deprived of carbon dioxide in Exp. II was very noticeable each day. The former invariably remained a pure yellow in colour while the latter developed a decided green colour. It is an accepted fact that etiolated shoots will not develop chlorophyll in the absence of oxygen in the air and thus the feeding test of Exp. III leads to another important conclusion. The formation of vitamin A in the plant must be independent of the presence of chlorophyll in the tissues (Fig. 3).

VI. INDEPENDENCE OF ULTRA-VIOLET LIGHT.

Another important conclusion may also be drawn from the foregoing experiments. The shoots in two cases, II and III, developed vitamin A when the light which they received had passed through two thicknesses of glass, one the ordinary window glass, the other the thick glass of the bell jar. Hence it may be concluded that the formation of vitamin A is independent of those ultra-violet rays of the spectrum which fail to pass through two thicknesses of ordinary glass—a point in which this process is similar to the more generally examined processes carried on in plant tissues.

VII. INFLUENCE OF CALCIUM SALTS.

The close relation between calcium and vitamin A in certain of the latter's activities led the writer to consider whether calcium played any part in the actual formation of the vitamin in the plant, and an attempt was made to cultivate shoots of *Tradescantia* in a medium devoid of calcium. This plant was chosen as it had previously been found very easy to cultivate in water cultures under laboratory conditions. About 200 cuttings were made from normal plants growing in profusion under the staging of a greenhouse. Each shoot, when prepared, consisted of about seven nodes and internodes, the terminal bud and four leaves, the lowest three leaves being cut off and the stem itself cut straight across immediately below the lowest node. The shoots were supported in corks in holes, the stems protected somewhat with a small covering of cotton-wool. Jars of 800 cc. or 1200 cc. capacity were used, four cuttings were put in the former, six in the latter, and the two lowest nodes

of each shoot were submerged in the solution. Half the cuttings were set in normal Sachs solution, half in a solution consisting of 1 g. KNO_3 , 0.5 g. NaCl , 0.5 g. NaH_2PO_4 , 0.5 g. MgSO_4 , and two or three drops of FeCl_3 sol. to a litre of distilled water. This last was obtained from an ordinary copper still, and was not distilled again. The jars were covered with brown paper, and placed on a window sill with a north aspect.

At first the shoots appeared to grow equally well in the two solutions, practically everyone of them taking root. But a striking difference between the roots was noticed on removing the paper to search for possible moulds. The roots in the Sachs solution had produced masses of root hairs throughout their length, while those in the solution without calcium were devoid of root hairs except for a short distance about half-an-inch long beginning about a quarter-of-an-inch behind each root tip. The root hairs never developed as far as those on the normal roots and apparently died away quite early. This confirms an observation made by Maquenne and Demoussy [1917, 1] in their work on the influence of calcium salts on the growth of seedlings (Plate I, Fig. 2).

When two or three more leaves had expanded on each shoot, the older ones were cut off, and later, when the root systems were well established, the plants were cut down so that only two nodes with undeveloped axillary buds were left. Practically all the plants survived this drastic treatment but the next shoots from the Sachs solution were distinctly more vigorous than those from the calcium-free solution. By this time, three months after the setting of the original cuttings, it was decided to begin the feeding test. 1 g. of the fresh shoot was given to each rat daily and although the shoots grown in the calcium-free solution did not give quite such good growth as those grown in the Sachs solution, yet the growth was very definite in two cases out of three. Maquenne and Demoussy [1917, 2] state that distilled water will dissolve out enough calcium from the glass of the culture vessels to supply the need of seedlings for some time. It would seem probable, however, that after three months with two changes of culture fluid, there would be only traces of calcium left, and it may be concluded that vitamin A can be produced in the presence of only the merest traces of calcium salts in the nutrient solution (Table I).

The question of examining the influence of other elements such as phosphorus, potassium, sulphur, etc., on the formation of the vitamin was also considered, but the difficulty generally experienced in getting cuttings to develop more than one or two more leaves (and no side shoots) in solutions free from these elements, individually, made the matter impossible. Briggs [1922] has just shown that the "photosynthetic activity of plants of *Phaseolus vulgaris* grown in culture solutions devoid of potassium, magnesium, iron or phosphorus is less than that of plants grown in full culture solution." Possibly a lack of calcium also retards photosynthetic activity which would account for the retarded growth of the *Tradescantia* cuttings in the calcium-free solu-

tion. It is impossible to contend that retarded photosynthetic activity in a leaf was the cause of the retarded formation of vitamin A as it has been shown that vitamin A can be formed under conditions ((a) lack of chlorophyll, (b) lack of CO₂ in atmosphere) in which photosynthesis (carbon assimilation) does not take place to any appreciable extent. And in fact, it is even open to question whether the observed difference in the response of the animals to the two sets of shoots (those grown with calcium and those grown without) was really significant or whether it lay within the limits of experimental error often observed in the only available and decidedly crude test at present used for the vitamin. The main point is that the vitamin can be formed in the presence of only very small traces of calcium in the nutrient solution.

SUMMARY.

1. Light is necessary for the formation of vitamin A in plant tissues.
2. This process can be carried on in the absence of carbon dioxide and of oxygen in the surrounding atmosphere.
3. It is independent of the presence of chlorophyll in the plant.
4. It is also independent of the ultra-violet rays of the spectrum (or such of those as are held back by two plates of window glass) and can be carried on under the influence of electric light in the absence of sunlight.
5. The presence of chloroform in the atmosphere prevents the formation of the vitamin.
6. The almost complete absence of calcium salts from the nutrient solution of a water culture of *Tradescantia* does not prevent the formation of vitamin A in the leaves of the plant.

It is a pleasure to express my gratitude to the Medical Research Council for a grant to defray the cost of this investigation.

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TRADESCANTIA CUTTINGS

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Fig. 1
Full Sachs Sol. Calcium lacking
Difference in Vegetative Growth



Fig. 2
Full Sachs Sol. Calcium lacking
Difference in Roots

XIX. THE ASSOCIATION OF VITAMIN A WITH THE LIPOCHROMES OF PLANT TISSUES.

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DURING the investigation of the conditions of formation of vitamin A in seedlings reported in the preceding paper, the association of vitamin A with lipochromes in plant tissues already emphasised by Steenbock and his co-workers (subsequent references) was being kept in view. Much of the earlier work on vitamin A consisted of the examination of such plant tissues as were commonly in use as food for human beings and farm stock. Steenbock and Gross [1919] found that, of all the roots and underground stems examined by them, the carrot and sweet potato were the only two that contained this vitamin; the other seven they described as a "complete failure." These latter included rutabaga, dasheen, red beet, parsnip, potato, mangold and sugar beet. In each experiment the root formed 15 % of the diet of the test animals, and in the case of the carrot and sweet potato, gave good growth. The minimum dose for growth of each of these was not determined, so that no exact comparison between the two was ever drawn. A rather more quantitative examination was made of the yellow and white maize seeds. When the former constituted 85 % of the ration, the test animals grew and reproduced themselves. The same ration of white maize showed a retardation of the rate of growth in varied lengths of time, which were dependent on the variety of maize used. It was also found that yellow maize in 48 % of the diet gave distinctly poorer growth than the higher ration.

Meanwhile, the close association of vitamin A with the lipochromes in animal fats led many workers to speculate on the possible identity of vitamin A with one of the lipochromes. That there was no foundation for this was, however, shown by Drummond [1919] who prepared pure crystalline carotene from carrots and demonstrated its complete lack of growth-promoting power. Later, also, Drummond and Coward [1920] and Palmer and Kennedy [1921] described many instances of animal fats which were rich in carotene and relatively poor in vitamin A, and others in which the reverse relation held. Steenbock, Sell and Bluell [1921] pointed out that the concentrations of pigment and vitamin A in butter were not closely parallel, though they were

in general agreement, and suggested that this might be due to their having the same source in the green food of the cow. Steenbock, Sell and Boutwell [1921] found that in six different varieties of dried peas, the greater the lipochrome content, the greater was the vitamin A content, though no definite comparison could be made as there is no method of measuring absolute quantities of the vitamin. Steenbock and Sell also found [1922] that the "white sweet potato and the white carrot contained little fat-soluble vitamin, which was in marked contrast to the yellow pigmented varieties. The tops of white carrot roots slightly pigmented with chlorophyll and containing a small amount of yellow pigment were found richer in fat-soluble vitamin than the bottoms containing one-half as much pigment. Green cabbage leaves taken from the heart of cabbage plants which failed to 'head' were found much richer in fat-soluble vitamin than white cabbage leaves in the head. The latter contained only one-tenth as much yellow pigment."

These observations made it seem desirable to examine the lipochrome content of the tissues used in the experiments reported in the previous paper [1923]. Accordingly, the wheat seeds and seedlings of experiment 1 were first tested. 20 g. of the seeds (460 in number) were saponified by heating on a water-bath with 50 % caustic soda for five days and with two volumes of alcohol for two days under a reflux condenser. The alcohol was then distilled off, the mass diluted with two volumes of water and extracted with light petroleum (B.P. 40–70°) three times. The extracts were combined, washed with water six times, always in an atmosphere of carbon dioxide, dried over anhydrous sodium sulphate, evaporated down in a current of carbon dioxide under reduced pressure and made up to 10 cc. exactly. The strength of the solution was estimated by means of a Hellige colorimeter, comparison being made against a 0.2 % solution of potassium dichromate. The concentration of the pigment was determined by reference to curves kindly lent by Dr O. Rosenheim and drawn according to observations made by himself on solutions of the lipochromes of known strength, in confirmation of Willstätter's work on the same point. The curves of carotene and xanthophyll are far from being identical: the curve for xanthophyll is more nearly a straight line than that for carotene. The estimation of the lipochromes in a solution cannot therefore be made exactly without first effecting a separation and then making an estimation of each independently. But where carotene and xanthophyll occur in about the same relative proportions in different tissues, one being largely in excess over the other, a fairly true comparison may be made by using the curve of the lipochrome which occurs in the greater proportion. To determine this proportion, Willstätter's phase test is applied. To a given volume of the light petroleum solution of the mixture, an equal volume of 90 % methyl alcohol is added. The xanthophyll goes into the lower alcohol layer, the carotene remains in the petroleum, and the relative strengths of the solutions may be roughly estimated. The amount of pigment in the seeds was extremely small, 0.02 mg. in the 460 seeds used, and, moreover, the pigment did not give the usual colour

reactions for lipochromes. Wheat seeds were also grown in the dark and light respectively for 12 days and the lipochrome content of the same number of shoots (460) estimated in the same way. In the 460 etiolated shoots were 1.062 mg. of lipochrome (carotene: xanthophyll = 1 : 3) estimated by Willstätter's curves as xanthophyll; while in the 460 green shoots were 1.875 mg. of lipochrome (carotene: xanthophyll = 1 : 3). Some yellow pigment was left in the remaining seeds and roots in both cases (0.064 mg. in the etiolated, and 0.1 mg. in the green), but it did not give the lipochrome reactions with strong sulphuric and nitric acids. The interesting point in the comparison is that the lipochrome content of both lots of shoots was very much greater than that of the seeds from which they had grown, and that the content of the green shoots was, roughly, nearly twice that of the etiolated shoots (Table I).

Etiolated and green shoots of yellow maize contained approximately equal amounts of lipochrome, which was greater than the amount contained in an equal number of seeds (Table I).

Table I.

	Weight in g.	No. of seeds or shoots	Lipochrome tests			Phase test C : X	Total lipochrome content mg.
			H ₂ SO ₄	HNO ₃	I in KI		
<i>Wheat:</i>							
Seeds	20	460	-	-	-		0.02?
Etiolated shoots		460	+	+	+	1 : 3	1.062
Green shoots		460	+	+	+	1 : 3	1.875
<i>Yellow maize:</i>							
Seeds	20	73	+	+	-	1 : 2	0.15
Etiolated shoots		73	+	+	-	1 : 3	0.21
Green shoots		73	+	+	-	1 : 3	0.21
<i>Peas:</i>							
Seeds, dry	26	126	+	+	-	1 : 3	0.11
Etiolated shoots		126	+	+	-	1 : 3	0.02
Shoots without CO ₂		126	+	+	-	1 : 3	0.11
Shoots without O ₂		126	+	+	-	1 : 2	0.09
Remainder seeds and roots		126	+	+	-	2 : 3	0.065

A similar comparison was made between seeds and shoots of peas treated as in experiments 2 and 3 of the preceding paper; the day on which the shoots were exposed to the light was however not nearly as sunny as any one during the feeding test. The results are summarised and it is interesting to note that there was less lipochrome in the etiolated shoots than in the seeds and the amount of lipochrome remaining in the roots and seeds (not nearly exhausted of the food store by this time) was larger than that of the etiolated shoots. The amounts in the shoots exposed to light (without carbon dioxide and without oxygen respectively) showed a content quite or nearly as great as the dry seeds (Table I). It is at least suggestive to note by the way that the ratio, xanthophyll/carotene, was less in the shoots deprived of oxygen than that in the shoots deprived of carbon dioxide. But the important point to note at present is that lipochromes are present in many of these tissues before the

appearance of the vitamin. A comparison between the absolute amounts of lipochrome present in shoots which will not promote growth and those which will do so once again proves that the activity of the tissue is not measured by its lipochrome content.

Lipochrome content of:

100 etiolated pea shoots	0.016 mg.	Inactive in dosage of one shoot			
.. pea shoots exposed to light in absence of CO ₂	0.09	Active
.. pea shoots exposed to light in absence of oxygen	0.07	"
.. etiolated wheat shoots	0.23	Inactive
.. green " "	0.4	Active
.. etiolated shoots of yellow maize	0.28	Inactive
.. green " "	0.28	Active

These estimations were made on much larger numbers of shoots than those indicated but have been reduced to 100 to draw the comparison of the lipochromes.

In the meantime, an examination was being made on all available lipochrome-containing tissues from plant growths as varied as possible from a botanical point of view; and a comparison was made according to lipochrome content, growth-promoting power, and calcium content (to verify if possible the finding reported in the preceding paper on the dispensability of calcium salts in the formation of vitamin A).

A polyanthus narcissus was first investigated. The perianth of this variety is a deep rich orange and the corona even darker, but an estimation of the lipochrome content showed that the whole perianth of six lobes contained only slightly more than the corona, a much smaller structure, of the same flower. No calcium was found in 3 g. of perianth, 2 g. corona or in 0.2 g. of the flower sheath; but 0.25 % of the dry weight of the stem was calcium, estimated on 2 g. fresh material. Twenty coronas given to each of two rats daily (put in a small dish in the cage and generally eaten greedily) produced good growth as did also the perianth lobes from 20 flowers to each of two other rats (Fig. 1 and Table III).

A pure "paper-white" narcissus gave slight growth in one of three rats, none in the other two; but an equal dose of the perianth and corona of a "poetaz" narcissus (white perianth and pale yellow corona) gave very definite growth in these same rats. No calcium was found in 3 g. of perianth of the paper-white narcissus though it formed 0.53 % of the dry weight of the stems (estimated on 6 g.) and 1.8 % of the dry weight of the brown sheaths (estimated on 0.4 g.) (Fig. 1 and Table III).

On giving coronas or perianths of a large daffodil (*Narcissus Buonaparte*) to rats, they dropped in weight at once, but on discontinuing this addition, they invariably recovered their previous weight and even grew for a few days. This suggested the presence of vitamin A in this tissue and also some toxic substance. To avoid the effect of the latter, the unsaponifiable matter was extracted from the various tissues and used for the following experiment. By a comparison of the vitamin content of daffodil buds with that of flowers opened in the laboratory (the stems being cut as short as possible) it was

hoped to obtain a confirmation of the previous finding on the formation of the vitamin in a plant tissue in the presence of lipochromes and in sunlight—that is, that there should be no question of its having been transported to the flowers from the leaves. Some growth was obtained on the unsaponifiable matter from 170 buds, but no growth was obtained on the extract from 140 flowers opened in the laboratory, nor on the extract from 140 flowers opened naturally. Possibly the conditions of its storage while waiting for suitable rats for the test accounted for the inactivity but it was too late in the season to repeat the test. Again no calcium was found in the buds (5 g.), in the flowers (5 g.) opened naturally, or in the flowers (6 g.) opened in the laboratory which had been supplied with North London tap-water. But the sheaths (1 g.), leaves (4 g.), and stems (6 g.) contained 0.57, 0.39, 0.39 % calcium, respectively, of their dry weights. This is interesting botanically as no stomata were found in these perianths or in those of any of the other narcissi, yet stomata occurred on the outer (botanically “under”) surface of the sheaths and in the stems and leaves. The epidermal cells of the perianths and coronas of all the yellow varieties had very definite conical-shaped outer walls, but they were not so highly developed in the white varieties. Haberlandt [1914] ascribes to this structure the function of concentrating the light on the contents of the cell (Tables II and III).

A chance of testing equally illuminated parts of the same flowers, the one containing much carotene and the other none, occurred in *Narcissus poeticus*—Barr’s “White Standard,” popularly known as “Pheasant’s Eye.” Seven hundred of these flowers were obtained, and the white perianths removed without the very small yellow patch that occurs at the base of each lobe.

The coronas were also removed and the two lots saponified separately. The unsaponifiable matter from the white perianth lobes (2.8 g. from 370 g. material) gave no growth in three rats, while the extract from the yellow coronas (0.2 g. from 11 g.) gave very good growth in three rats for eleven days (Fig. 1).

Yellow tulips gave growth on the unsaponifiable matter of their leaves and perianths, the former being stronger than the latter. Again no calcium was found in the perianths though it formed 2.5 % of the dry weight of the leaves. The stems contained no detectable calcium and in this flower there is no sheath. The lipochrome content of the perianths was low, being only 0.005 mg. in 10 g. fresh material (Tables II and III).

Other instances of the association of vitamin A with lipochromes¹ in plant tissue studied in the work are afforded by (a) tomato pulp, (b) cucumber skin, (c) yellow iris flower, the sepals, petals, petaloid stigmas being used in turn, but *not* the stamens or pollen, (d) orange juice, (e) red capsicum fruit, (f) yellow capsicum fruit, (g) the calyx of the ripened fruit of the winter cherry (Tables II and IV). In this connection, it should be noted that tomato

¹ A full account of the lipochromes (carotinoids) and their occurrence in animal and plant tissues has just been written by Palmer (1922).

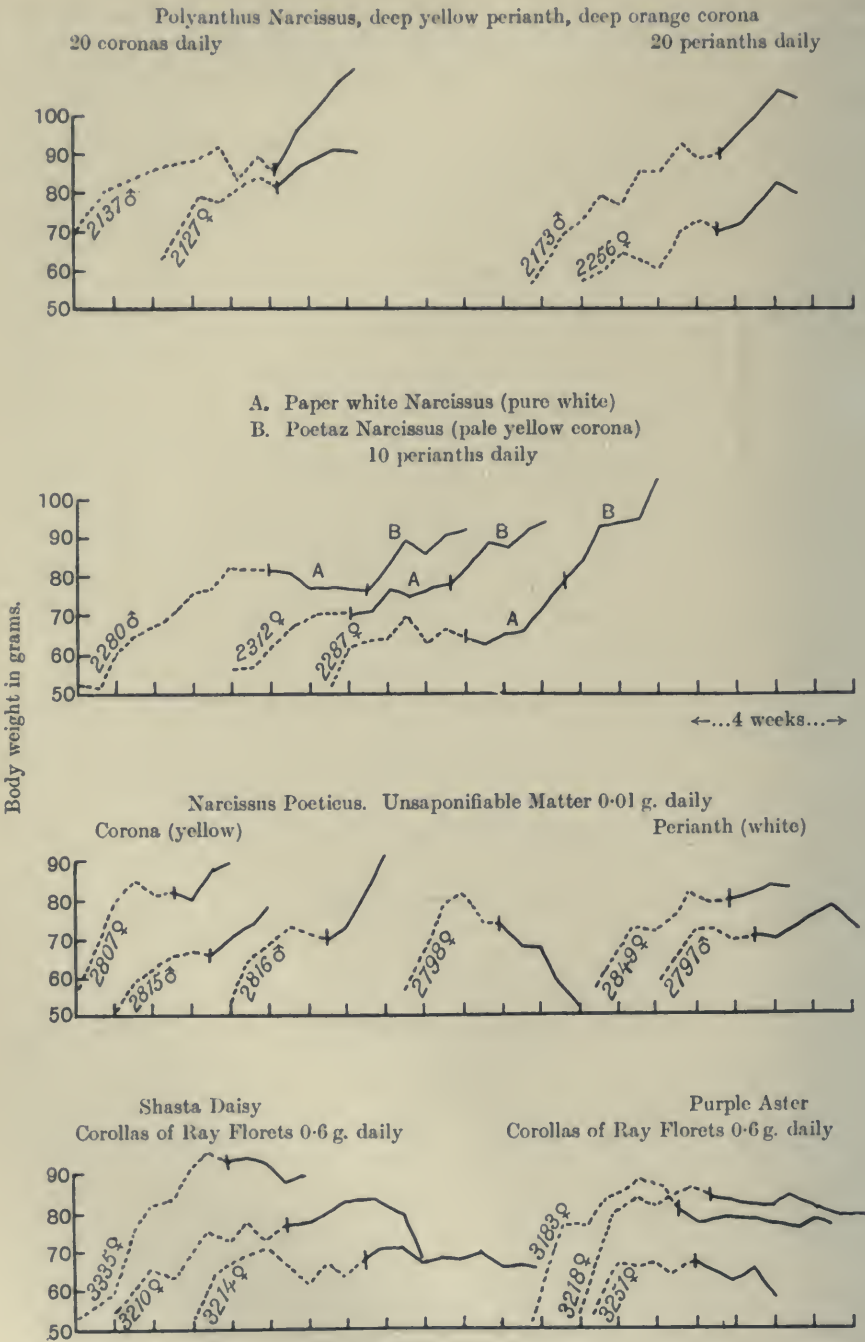


Fig. 1.

Preparatory period on A-deficient diet shown in dotted lines.

fruit has already been shown to contain vitamin A by Osborne and Mendel [1920, 1], that indications of the presence of the vitamin in orange juice have been noted by Osborne and Mendel [1920, 2] and also by Hess, McCann and Pappenheimer [1921].

The absence of vitamin A is associated with absence of lipochrome in (a) four varieties of mangolds (the flesh of the root only tested), (b) one variety of swede, (c) the fleshy part of the cucumber fruit (slight), (d) the inflorescence of the cauliflower, (e) the corollas (only) of the ray florets of the Shasta daisy, (f) the corollas (only) of the ray florets of a purple aster (Tables II and IV, and Fig. 4).

Table II.

	Daily dose. Unsap. matter	Body weight of rat in g.				
		0	7	14	21	28 days
<i>Narcissus Buonaparte:</i>						
Perianths of buds	0.01 g.	72	72	82	82	—
		82	89	90	94	—
		98	100	120	124	—
						} Very fair
Perianths of flowers opened naturally	0.01 g.	90	89	—	—	—
		70	62	—	—	—
		84	95	—	—	—
						} Probably none (period too short)
Perianths of flowers opened in lab.	0.01 g.	99	97	97	—	—
		76	73	76	—	—
		72	76	70	—	—
		81	81	65*	—	—
						} None
Leaves	0.01 g.	74	85	94	—	—
		102	126	142	—	—
		89	92	112	—	—
						} Very good
<i>Yellow Tulip:</i>						
Perianth	0.01 g.	76	74	82	90	—
		81	89	100	87 (cold)	—
		75	74	76	77	—
						} Slight
Leaves	0.01 g.	81	86	102	105	—
		69	70	79	86	—
						} Fairly good
	Fresh matter					
<i>Tomato pulp</i>	0.5 g.	88	97	116	125	130
		79	80	92	99	102
		82	88	86	95	99
						} Fairly good
<i>Cucumber skin</i>	1.0 g.	96	109	116	122	130
		79	91	110	112	112
		62	60	70	73	70
						} Good
<i>Yellow iris, sepals, petals and stigmas</i>	0.2 g.	74	83	—	—	—
		69	74	—	—	—
		83	101	—	—	—
						} Probably good (period too short)
<i>Orange juice</i>	10 cc.	96	103	111	—	—
		85	100	115	—	—
		83	97	112	—	—
						} Good
<i>Red capsicum, fruit</i>	1.0 g.	94	102	116	—	—
		74	82	99	—	—
						} Good
<i>Yellow capsicum, fruit</i>	1.0 g.	91	98	116	—	—
		96	111	121	—	—
						} Good
<i>Winter Cherry, calyx only</i>	0.1 g.	92	103	115	—	—
		78	84	90	—	—
		83	96	107	—	—
						} Good

Table II *continued*

	Daily dose Fresh matter	Body weight of rat in g.					
		0	7	14	21	28 days	
<i>Mangolds</i> : Golden Tankard	1.0 g	98	95	78	—	—	None
		96	109	94	—	—	
		90	88	79	—	—	
<i>Sweedes</i> : Red Queen	1.0 g.	75	78	84	—	—	None
		72	75	73	—	—	
		83	84	78	—	—	
<i>Cucumber</i> , fleshy part of fruit	1.0 g.	89	91	90	98	98	Slight
		75	78	84	91	86	
<i>Cauliflower</i> , inflorescence	1.0 g.	96	98	100	—	—	None
		78	81	80	—	—	
	Unsap. matter						
<i>Laminaria</i>	0.01 g.	104	118	113	—	—	Practically none
		105	105	108	—	—	
		105	105	105	—	—	
<i>Fucus vesiculosus</i>	0.01 g.	93	89	102	—	—	Probably none
		76	65	66	—	—	
		77	72	66	—	—	
<i>Lilium candidum</i> , pollen	0.01 g.	80	82	79	—	—	None
		82	92	88	—	—	
		83	88	83	—	—	
<i>Carrot</i> shoots, etiolated	0.8 g.	97	103	106	—	—	Fair
		90	95	101	—	—	
		82	91	91	—	—	
<i>Carrot</i> shoots, etiolated	0.2 g.	89	93	96	—	—	Fair
		85	91	97	102	—	
		97	111	119	119	—	
<i>Turnip</i> shoots, etiolated	0.2 g.	94	94	92	—	—	None
		68	70	66	—	—	
		72	70	63	—	—	
<i>Carrot</i> , roots	0.01 g.	63	63	78	—	—	Fairly good
		56	62	66	—	—	
		61	63	71	—	—	
<i>Turnip</i> , roots	0.01 g.	96	78	65	—	—	None
		86	78	81	—	—	
		71	66	61	—	—	
<i>Polygonthus narcissus</i> , stems	0.01 g.	102	105	—	—	—	Fair
		66	71	72	—	—	
		75	80	86	—	—	

An effort was made in the late summer to find a yellow flower whose pigments were wholly water-soluble, but in five varieties of *Nasturtium*, in *Helianthus annuus*, in the African marigold, in *Ranunculus acris*, in *Lotus corniculatus*, lipochromes were always present. Hence no example of the association of the vitamin with a yellow water-soluble pigment has been traced, and its non-occurrence in the flesh of the mangolds and swedes which contain large quantities of these pigments has been clearly demonstrated, though in this case, of course, light has had no immediate influence.

Another apparent exception to what appears to be a general rule, the association of vitamin A with lipochromes wherever they are exposed to light, was found in *Fucus vesiculosus*, a brown seaweed which grows just below high water mark; patches of this are mingled with patches of *Enteromorpha*, a green

Table III.

					Water	Ash % of dry wt.	Calcium		Dose g.	Growth
							% of dry wt.	estimated on approx. g.		
<i>Polyanthus narcissus</i> :									Fresh matter	
Perianth lobes	90.96	9.5	0	3	1	Good
Coronas	86.61	5.82	0	2	0.5	"
									Unsap. matter	
Stems	93.90	6.23	0.25	2	0.01	Slight
<i>"Paper-white" narcissus</i> :									Fresh matter	
Perianth including corona	89.49	8.17	0	3	1	Very slight
Stems	90.42	8.3	0.53	6	—	—
Flower sheaths (brown)	12.76	7.7	1.8	0.4	—	—
<i>Narcissus Buonaparte</i> :									Unsap. matter	
Perianths of buds	83.57	3.65	0	5	0.01	Fair
Perianths of flowers opened naturally	89.93	4.55	0	5	0.01	None
Perianths of flowers opened in lab.	92.76	6.45	0	6	0.01	"
Flower sheaths (brown)	49.78	7.35	0.57	1.0	—	—
Leaves	85.87	7.0	0.39	4	0.01	Very good
Stems	92.74	6.05	0.39	6	—	—
<i>Yellow Tulip</i> :										
Perianth	89.45	5.32	0	3	0.01	Fair
Leaves	87.56	9.64	2.52	3	0.01	Good

(It is interesting to note incidentally the occurrence of calcium in those tissues in which photosynthesis (carbon assimilation) has at some time taken place and its apparent absence from those tissues where presumably this process has never been carried on.)

seaweed, on the boulders and rocks of the seashore, but on the groynes built out as breakwaters on the South Coast, the *Fucus* grows distinctly below the green weeds and therefore would be covered by the tide for a somewhat longer period than the green ones. A test on the washed fresh material previously reported [Coward and Drummond, 1921] gave no evidence of vitamin A in *Fucus*, though a green weed *Enteromorpha* had given positive results, and later tests on the extract of unsaponifiable matter of *Fucus vesiculosus* and *Laminaria*, respectively, have also detected no vitamin (Table II). The lipochromes of this species and of *Laminaria* were then examined, the method adopted being as follows.

The fresh material was ground up with solid caustic soda (about 1 g. to 5 g. fresh material) to break down the chlorophyll into components (chlorophyllins etc.) which are insoluble in light petroleum, and also to prevent possible enzyme action. A few drops of ether were also added, and, when necessary, a little silver sand to help to break down the tissue. The mixture was then ground up with anhydrous sodium sulphate to retain water, and light petroleum (B.P. 40-70°) added to dissolve out the lipochromes. In the case of *Laminaria*, the extract was scarcely coloured and a further extract with the same solvent was colourless. Ether was then tried and formed a deep yellow solution which gave a well-defined spectrum 475-450 and 420 to the end. There was only a slight indication of the most characteristic carotene band, i.e.

490-480. The solution gave the light blue colour reaction with 30 % hydrochloric acid characteristic of fucoxanthin according to Willstätter—and also the general lipochrome reactions with conc. H_2SO_4 and HNO_3 . A second ether extract of the same preparation gave a spectrum with no trace of the first carotene band, but gave the fucoxanthin bands very well defined again. It was concluded that *Laminaria* contained only the merest traces of carotene or xanthophyll, but large quantities of fucoxanthin.

Table IV

	Daily dosage g.	Lipochrome present	Spectrum	Colour reactions with		Activity
				H_2SO_4	HNO_3	
Tomato pulp	0.5	Lycopin carotene?	Typical Masked by lycopin if present	+	+	Good
Cucumber skin	0.5	Carotene and xanthophyll	Typical	+	+	"
Yellow Iris, flower	0.2	"	"	+	+	"
Orange juice	10 cc.	Carotene and xanthophyll 4 : 1	"	+	+	"
Red capsicum, fruit	0.1	Lycopin carotene? no xanthophyll	"	+	+	Very good
Yellow capsicum, fruit	1.0	Carotene, very little xanthophyll	"	+	+	" "
Winter Cherry, calyx	0.1	All carotene	"	+	+	Good
No attempt was made in this test to find the minimum dosage for growth.						
<i>Mangolds</i> (flesh of root):						
Sutton's Golden Globe	1	—	—	—	—	Poor
Carter's Red Chief	1	—	—	—	—	None
Carter's Imp. Long red	1	—	—	—	—	"
Toogood's Golden Tankard	1	—	—	—	—	"
<i>Swede</i> (flesh of root):						
Sutton's Red Queen	1	—	—	—	—	"
Cucumber, fleshy part of fruit	1	Little carotene and xanthophyll	+	+	+	Slight
Cauliflower, inflorescence	1	—	—	—	—	None
Shasta daisy, corollas of ray florets	0.6	—	—	—	—	"
Purple aster, corollas of ray florets	0.8	—	—	—	—	"

(The colour reactions were obtained by drying a little of the light petroleum solution on a glazed tile and adding one drop of the acid. The colour was observed at the edge of the drop of acid before charring took place.)

Fucus, on the other hand, gave a rather stronger solution of lipochromes with light petroleum; carotene or xanthophyll and fucoxanthin (traces only) were identified by means of the spectrum and 30 % HCl reaction, respectively. A further extraction with ether took out far more pigment and gave the carotene spectrum and the fucoxanthin reaction very strongly. This was repeated several times, and always the two lipochromes were obtained. A phase test of the light petroleum solution with MeOH gave a colourless lower layer, so that xanthophyll is not always present, though Willstätter and Stoll [1913] record xanthophyll and no carotene.

Pelvetia behaved like *Laminaria*, containing much fucoxanthin and very little indeed of carotene or xanthophyll.

Thus, although lipochromes are present in two brown seaweeds, fucoxanthin in *Laminaria* which is exposed only at low tide, and fucoxanthin and carotene or xanthophyll in *Fucus*, and both are exposed to a certain amount of sunlight daily, yet neither has apparently produced any vitamin A detectable by feeding tests. The specimens were examined in late summer, when the intensity of the sunlight would have been great enough in other plants, and the exposure to the sunlight would probably have been long enough, at least for the *Fucus*. The penetration of the light rays into the tissues of seaweed must be much hindered by the thickness of the cuticle and it is conceivable that the light may be adequate for the process of carbon assimilation but not adequate or not of the right quality for the formation of vitamin A.

An exception, unexplainable at present, was found in the pollen of *Lilium candidum*. This contained large quantities of carotene (no xanthophyll by the phase test) which gave a typical spectrum and the usual lipochrome reactions. The ripe pollen from four stamens was rubbed on to a small piece of diet each day and given to each test rat. A week of this test gave very little growth and the pollen began to decompose; the remainder was pounded up with anhydrous sodium sulphate extracted with light petroleum and the extract evaporated down, mixed with a little inactivated olive oil and a drop of this solution (about 50 %) used as the daily ration. This resulted in no further growth and it was decided that the pollen was probably inactive, but the test will be repeated next summer (Table II).

THE ASSOCIATION OF VITAMIN A WITH LIPOCHROMES IN TISSUES NOT UNDER THE INFLUENCE OF LIGHT.

The classic example of this association is the root of the carrot, which has long been known to contain appreciable quantities of vitamin A. Its lipochromes are almost entirely carotene. The sweet potato is also reported by Steenbock as containing both carotene and vitamin A, and yet these two structures are almost completely in the dark, during their growing period. It does not seem probable that their subsequent exposure to variable and often very dull light after removal from the soil would bring about the formation of the relatively large quantities of vitamin A reported in these tissues.

A confirmation of this association in the carrot was made on the unsaponifiable fraction obtained in the usual way, and at the same time the corresponding fraction from turnips was prepared. The carrot extract used in a dosage of 0.01 g. produced very definite growth, the turnip extract produced none at all (Table II).

The question arose whether it was possible that the vitamin had been formed *in situ* in the root of the carrot or whether it had been transported

there from the leaves, where it is known to occur (earlier references). It is, of course, impossible to cut off the current of organic matter passing down to the root of the plant without at the same time preventing the development of the root; and, although not a strictly comparable process, it was decided to see whether the vitamin in the root could be transported upwards to leaves grown from carrots in the dark. Carrots were therefore cut off about an inch from the base, the cut ends placed in water and allowed to sprout. The leaves grew with very long stems, often 5 ins. long, and very small blades, never more than $\frac{3}{8}$ in. in diameter and mostly less. The age of these shoots could not be controlled as the age of the etiolated seedlings had been, so that the material used for the feeding test was from 3 to 5 weeks old. A dose of 0.1 g. of this fresh material made no impression on the growth of the test animals, but 0.2 g. produced a definite resumption of growth. A contrast to this result was obtained by testing 0.2 g. of the leaves of turnips sprouted in the dark: this dosage gave slight growth in one rat, none at all in three (Table II). The shoots of the carrot contained carotene, as shown by the spectrum, phase test, and lipochrome reactions, and no xanthophyll; the turnip shoots contained no lipochrome. Hence, it appears that the vitamin can be transported upwards at least, and when this occurs carotene seems to be transported also. Vitamin has been found in the green stems of polyanthus narcissus flowers (Tables II and III), but the stems contained lipochromes, the epidermis had stomata and assimilatory tissue and hence was comparable to the leaf of the plant. Hence, until more accurate investigation of the different regions of these stems has been carried out, it is not possible to draw any conclusion from the last observation.

The chief point in the formation of the vitamin which is apparent from these experiments is that some lipochrome (generally carotene) is always associated with the vitamin in plant tissues; and that where carotene is found, particularly carotene exposed to sunlight, there the vitamin may be expected to be present also.

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XX. STUDIES IN ANAEMIA. I.

THE INFLUENCE OF DIET ON THE OCCURRENCE OF SECONDARY ANAEMIA FOLLOWING REPEATED HAEMORRHAGES IN RATS.

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AIMS.

It seems a very obvious theorem that repeated haemorrhages would produce a chronic anaemia much more easily in an animal fed on a poor diet which lacks food factors essential for the formation of haemoglobin, such for instance as iron, than in one fed on a satisfactory and complete diet, and the first part of the following research was undertaken to demonstrate this. In practice several limitations of this principle are found. Thus one of the unpurified diets poorest in iron, white bread and whole milk, contains some materials for blood formation. Moreover there is a marked tendency even on a comparatively iron-poor diet to storage of a reserve of blood forming materials. Further, a limitation in the contrary direction is that a diet which is adequate in a fully grown animal to prevent an anaemia is inadequate in a similarly bled growing animal, presumably because it has not only to maintain its blood mass but also to increase it. These points are brought out in the following work.

PRELIMINARY WORK ON CATS.

When this work was begun cats were chosen in preference to rats because it was easy to withdraw blood repeatedly from them by cardio-puncture under an anaesthetic. This avoided open operation and the animals were perfectly well in 24 hours. Five cats were fed on bread, milk and fat and were subjected every week to bleedings amounting each to 1% of the body weight. Controls were fed on the same diet but not subjected to bleeding. Other controls were given meat in addition to the bread and milk and were subjected to similar haemorrhages. The idea underlying the research was to find out if the first set of cats could be brought to a stage where they exhibited a chronic anaemia characterised by a fall in the colour index of the corpuscle. This was the case. The colour index of the cats fed on bread and milk and bled maintained itself or even increased at first, but then fell to sub-normal. The cats which were fed on bread and milk maintained their colour index. The controls which

were to be given meat and bled gave considerable trouble, as an infectious gastro-intestinal disease broke out amongst them and the mortality amongst them and the difficulty of stamping it out led eventually to my abandoning the use of these animals and substituting rats. The difficulty of bleeding rats repeatedly, which had previously been an obstacle to their use, was solved by having recourse to leeches, as will be described later.

OUTLINE OF WORK ON RATS.

Rats are suitable animals for this work, because so much research on diets has been done on them. They were bred from mothers which had been fed before and during pregnancy and weaning on a liberal mixed diet.

In the inception of the work unpurified diets were used, the basal diet being white bread and whole milk and the ration the ample one of 20 g. white bread and 75 cc. whole milk per 100 g. rat per day. In addition certain controls were given from the time of weaning a liberal supply of green stuff, cabbage, etc., to the amount of 10 g. per 100 g. rat per day. The actual amount of green food eaten is not known but it was certainly one-half and may have been as much as three-quarters of the green food given. The rats exhibited marked preference for the tender parts of plants, *e.g.* the heart leaves of cabbage. Thus the two diets differed only in the presence or absence of green stuff but the difference in the result might be assigned to one or more of several factors:

1. *Differences in iron content.* The results of different observers taken from Sherman [1907] are given in tabular form.

Bread.

Observer	Iron per 100 g. mgm.			
Stockman	0.6	Fine bread
"	0.4	Common bread
Sherman	0.7	
Bunge	0.7	Minimum
"	1.0	Maximum
Average 0.7				

Milk.

Bunge (1875)	0.24	
" (1904)	0.30	
Abderhalden	0.23	
Stockman	0.33	Average of 3 estimations
Sherman	0.24	" 5 "
Average 0.27				

Thus there is fairly general agreement about the iron content of bread and milk. Taking the averages given above this would make in the bread and milk diet 0.35 mgm. iron per 100 g. rat per day if the whole of the bread and milk was eaten (which was not the case).

There is no such convincing similarity between the figures given for the iron content of green vegetables by different observers.

Green vegetables.

Material	Observer	Iron per 100 g. mgm.
Spinach	Boussingault ¹	4.5
"	Sherman	2.8 ²
"	Bunge	3.2 ²
"	Baldoni	2.0
Cabbage	Boussingault ¹	3.9
" (outer green) ...	Hausermann	1.4
" (inner yellow) ...	"	0.4
" ("edible portion")	Sherman	0.9
Lettuce ³	"	0.3

¹ This observer's figures are regarded by Stockman as too high.

² Corrected as advised by Sherman "Iron in Food."

³ Presumably the edible portion, i.e. the heart.

It would appear unsound to calculate from any average of these data the iron content of the mixed green stuffs given, but it is certain that the diet with green stuffs contains additional iron and it is probable that the iron is in some different form.

2. *Differences in vitamin content.* Bread and milk is usually recognised as containing a bare sufficiency of vitamins but green stuff contains a liberal supply of them.

3. *Differences in other substances.* Other mineral salts, organic groupings such as might be contained in chlorophyll, etc.

From a few days after weaning the experimental animals were fed on the basal diet alone and shortly after puberty, i.e. at the age of 90 days, they were subjected to a series of eight bleedings, each of about 1% of the body weight, at intervals of a week. The question was whether these animals would develop a more or less chronic anaemia characterised by a low colour index.

The controls were: (a) animals fed on the basal diet plus green stuff, these animals being subjected to an identical series of bleedings; (b) animals fed on the basal diet alone and not subjected to bleeding.

In consequence of preliminary experiments it was found necessary to start differential feeding at a very early age because indefinite results were obtained if the rats were fed on a mixed diet (with green stuff) till puberty and differentiation in the diet was delayed till then. The rats which were started on bread and milk only after puberty required a considerable number of these small bleedings (12 or more) before they showed any fall of colour index.

PRELIMINARY DISCUSSION.

At this early stage attention may be called to two papers which appeared while this work was in progress. It will be noted that the blood drawn on each occasion in this work was equal to 1% of the body weight; Geiling and Green [1921] showed that after a haemorrhage of double this size regeneration was complete in 7-10 days. It was therefore reasonable to expect that in this experiment between haemorrhages regeneration would take place to a considerable extent if not completely. The same authors, using purified diets,

showed that a diet deficient in protein, vitamin or mineral matter appreciably delayed blood regeneration after two haemorrhages on successive days.

Jencks [1922] showed that protein causes more rapid regeneration than fat or carbohydrate; vitamin-rich food is specially valuable for blood regeneration.

The work which is detailed below bears out these results but also an intrinsic value may be claimed for it inasmuch as the problem is approached from a slightly different point of view; these observers noted the rate of recovery from an acute experimental anaemia on different diets; this work demonstrates the complementary proposition that a series of haemorrhages insufficient to cause an anaemia (as judged by the lowering of the colour index) on the rich diet is sufficient to cause a moderately persistent anaemia on the poor diet provided growing animals have been kept long enough on it to reduce their reserve stores of blood forming materials to a minimum.

DETAILS OF EXPERIMENTAL PROCEDURE.

Method of bleeding. As fatty changes had been observed in the liver and kidneys in the experiments on cats it was thought wise to avoid anaesthetics, which themselves cause these changes. If the animals were bled from the tail it was a little difficult to control the amount abstracted and resort was made to the use of leeches. The rat was clipped with artery forceps in a piece of sacking; an area on the flank was shorn and a snick was made in the skin with a pair of scissors. To this the leech was applied. This proved an admirable way of extracting blood—for by this means a large number of profuse bleedings can be made on the same rat without trouble. A little experience enabled one to judge approximately from the degree of swelling of the leech how much blood had been abstracted and the exact quantity was determined by the difference in its weight before application and after removal.

Samples of blood, which flowed freely from the incision until stopped by pressure, were taken and examined by the haemocytometer and haemoglobinometer, the apparatus used being those of Bürker and Gowers respectively.

Attention was paid to the following points in the use of the haemocytometer.

1. A fair sample of blood was taken. The blood was allowed to flow freely and the haemocytometer and haemoglobinometer blood was taken so far as possible from the same drop.
2. Pipettes and counting chamber were standardised.
3. The cover glass and Bürker slide were polished till they adhered over parts of the areas in contact, Newton's rings being obliterated there.
4. Solution of corpuscles in the counting fluid (which occurs in Toison and sodium citrate solutions in the case of anaemic bloods) was avoided by using instead mammal Ringer to every 100 cc. of which 3 cc. formalin were freshly added.

5. The mixed fluid was run quickly and evenly through the Bürker chamber. Delay leads to the count being high, as also does any great overflow into the trough.

6. At first 256 squares in each Bürker chamber were counted, but later it was found sufficient to count two sets of 64 squares each in each chamber.

As regards the haemoglobinometer, Gower's standard was finally selected in preference to Haldane's as it was found that its correction constant when tested against a blood of known oxygen capacity remained the same, being 100/83.3 at the beginning of the experiments and 100/83.5 at the end. In the same time the correction constant for the Haldane standard fell from 100/86.5 to 100/92.3, indicating that the standard had faded.

The colour index is expressed in terms of the human standard, this being taken as 1. It is obtained by dividing the percentage of haemoglobin by twice the number of red blood corpuscles.

Tables and a chart showing the results of the experiment follow.

Table I. *Showing the blood condition of normal adult rats on mixed diet.*

Haemoglobin %	Red blood corpuscles in millions per mm. ³	Colour index (human index = 1)
81.5	7.34	.56
103	8.74	.59
96	8.57	.56
117	10.6	.55
100	9.03	.55
103	9.27	.56
103	9.27	.56
93	8.40	.55
114	9.63	.59
105	9.53	.55
99	8.90	.56
72	6.50	.55

Average colour index = .56.

Table II. *Showing the blood condition of control rats put on a diet of bread and milk after weaning and not subjected to bleedings.*

Age in weeks	Haemoglobin %	R.B.C. in millions per mm. ³	Colour index (human = 1)	Average C.I.
14	100	9.06	.55	.56
	112	10.08	.56	
	102	8.32	.61	
	100	8.85	.56	
	100	9.16	.54	
	105	9.56	.55	
18	95	8.70	.55	.54
	107	9.26	.58	
	95	8.90	.53	
	90	8.75	.51	
22	93	8.70	.53	.53
	100	9.48	.53	
	102	8.78	.58	
	107	10.76	.50	
26	95	9.25	.51	.51
	98	9.80	.50	
	102	9.80	.52	

Table III. *Showing blood condition of rats put on a diet of bread and milk at weaning and at the age of 13 weeks subjected to a series of 8 weekly haemorrhages.*

Rat 1.

Age in weeks	Bled cc.	Hæmoglobin %	R.B.C. in millions per mm. ³	Colour index (human = 1)
14	1.2	90	7.63	.59
15	1.2	76	5.91	.64
16	1.0	67	6.42	.52
17	1.4	54	5.92	.46
18	1.3	39	4.75	.41
19	1.1	51	7.80	.33
20	1.6	51	7.35	.35
21	1.3	—	—	—
22	Not bled	51	7.30	.35
23	"	56	8.07	.35
24	"	—	—	—
25	"	88	11.55	.38
26	"	88	9.68	.46
27	"	102	9.65	.53

Average weight during period of bleeding 116 g.

Average amount of blood taken 1.3 cc. = 1.12 % of body weight.

Rat 2.

14	1.4	93	8.16	.57
15	1.5	78	6.26	.62
16	1.3	88	8.04	.55
17	1.8	54	4.86	.56
18	1.1	49	5.98	.41
19	1.3	44	5.24	.42
20	1.3	26	4.22	.31
21	1.4	42	5.56	.37
22	Not bled	54	8.03	.34
23	"	59	7.48	.40
24	"	—	—	—
25	"	83	10.64	.39
26	"	90	10.36	.44
27	"	100	9.85	.51

Average weight during period of bleeding 129 g.

Average amount of blood taken 1.4 cc. = 1.08 % of body weight.

Rat 3.

14	1.3	85	7.00	.60
15	1.2	78	5.84	.67
16	1.5	93	7.92	.59
17	1.3	58	5.53	.53
18	1.0	44	5.40	.41
19	.9	41.5	5.28	.39
20	1.7	49	6.46	.38
21	1.3	44	5.65	.39
22	Not bled	49	6.62	.37
23	"	54	7.54	.36
24	"	—	—	—
25	"	99	10.62	.46
26	"	—	—	—
27	"	100	9.07	.55

Average weight during period of bleeding 137 g.

Average amount of blood taken 1.3 cc. = 1 % of body weight.

Rat 4.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm. ³	Colour index (human = 1)
14	1.1	98	8.40	.58
15	1.5	—	—	—
16	1.5	83	6.05	.68
17	1.3	68	6.40	.56
18	1.2	73	6.27	.58
19	1.7	54	5.00	.54
20	1.5	49	5.64	.43
21	1.8	59	6.55	.43
22	Not bled	60	8.12	.37
23	"	63.5	9.82	.32
24	"	—	—	—
25	"	93	11.56	.41
26	"	—	—	—
27	"	98	9.38	.52

Average weight during period of bleeding 144 g.

Average amount of blood taken 1.5 cc. = 1.04 % of body weight.

Rat 5.

14	1.0	98	8.35	.59
15	1.1	—	—	—
16	1.0	—	—	—
17	1.2	73	6.63	.55
18	1.5	49	5.00	.49
19	1.3	61	6.10	.50
20	1.4	66	8.45	.39
21	1.4	46	6.15	.37
22	Not bled	41.5	5.94	.35
23	"	44	7.35	.30
24	"	—	—	—
25	"	100	10.70	.47
26	"	—	—	—
27	"	100	9.55	.52

Average weight during period of bleeding 112 g.

Average amount of blood taken 1.3 cc. = 1.16 % of body weight.

Table IV. *Showing the blood condition of control rats fed on bread and milk and green stuffs and at the age of 13 weeks subjected to a series of 8 weekly haemorrhages.*

Rat A.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per mm. ³	Colour index (human = 1)
14	1.0	98	8.46	.58
15	1.1	107	8.52	.63
16	1.1	84	7.36	.57
17	1.3	88.5	8.20	.54
18	1.2	84	7.62	.55
19	1.6	84	7.78	.54
20	1.5	94	8.40	.56
21	1.9	98	8.92	.55
22	1.9	90	8.06	.56

Average weight during period of bleeding 143 g.

Average amount of blood taken 1.4 cc. = 1 % of body weight.

Rat B.

Age in weeks	Bled cc.	Haemoglobin %	R.B.C. in millions per min. ³	Colour index (human = 1)
14	1.1	110	9.33	.59
15	1.1	91	7.35	.62
16	1.3	98	8.42	.58
17	1.2	86	7.56	.57
18	1.1	89	8.26	.54
19	1.3	95	8.66	.55
20	1.9	93	8.20	.57
21	1.9	91	8.22	.55
22	2.0	91	8.32	.55

Average weight during period of bleeding 140 g.

Average amount of blood taken 1.4 cc. = 1 % of body weight.

Rat C.

14	.9	108	9.33	.58
15	1.3	93.5	9.16	.51
16	1.4	84	8.42	.50
17	1.4	103	9.38	.55
18	1.2	98	9.06	.54
19	1.3	95	8.80	.54
20	1.8	93	8.48	.55
21	1.7	98	9.07	.54
22	1.5	98	9.00	.54

Average weight during period of bleeding 140 g.

Average amount of blood taken 1.4 cc. = 1 % of body weight.

Rat D.

14	.8	105	8.74	.60
15	.9	103	7.98	.65
16	1.4	98	8.40	.58
17	1.5	95	7.68	.55
18	1.2	98	8.64	.57
19	1.4	93	8.76	.53
20	1.7	88	7.96	.55
21	1.9	88	8.10	.54
22	1.3	86	8.08	.53

Average weight during period of bleeding 139 g.

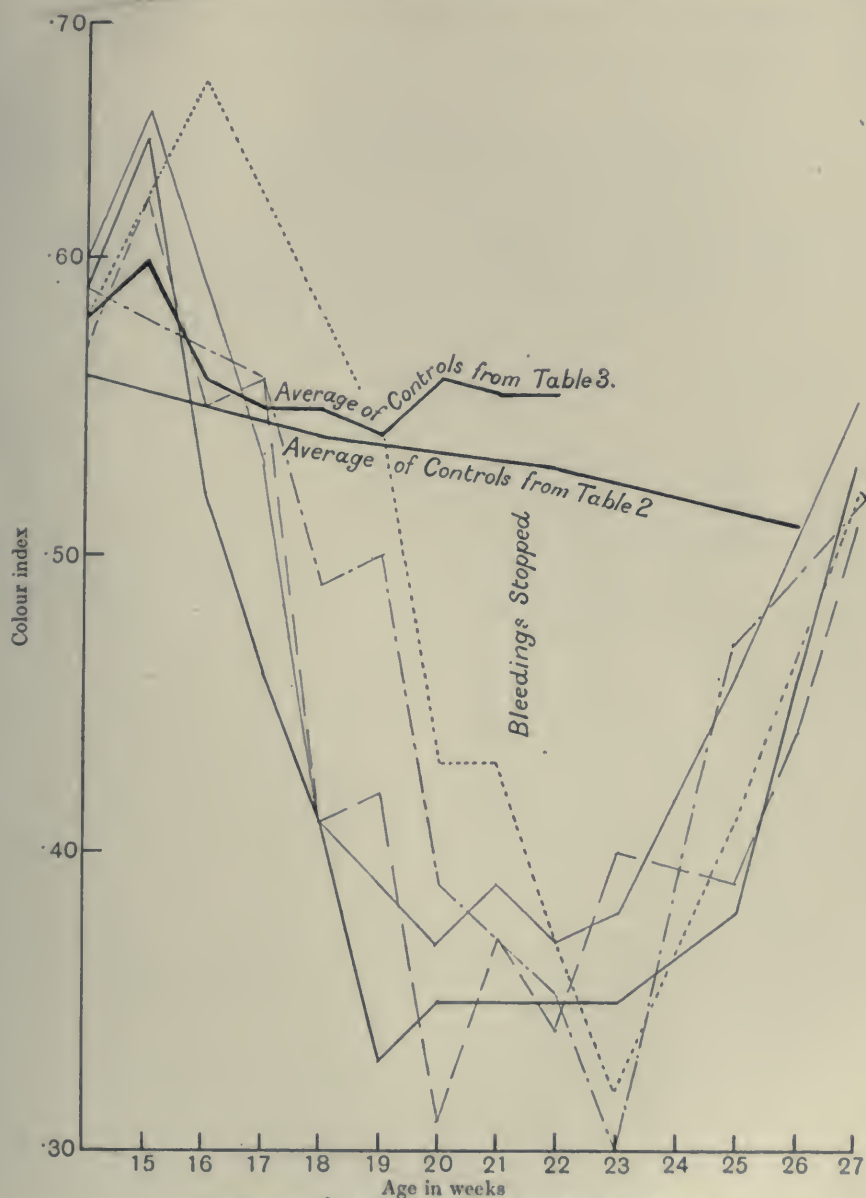
Average amount of blood taken 1.3 cc. = 1 % of body weight.

It is to be noted that the first bleeding was followed by a rise in the colour index instead of by a fall. This increase in colour index has been specially noticed by Hough and Waddell [1916].

CONCLUSION.

Rats fed from the time of weaning on white bread and whole milk and subjected to a series of eight bleedings each amounting to 1 % of the body weight show a fall in colour index which persists for 2-3 weeks after the bleeding is stopped; whereas controls fed from the time of weaning on this diet plus green stuff show a comparatively inappreciable fall of colour index. Controls fed on this diet but not bled also show a comparatively inappreciable fall of colour index.

The receipt of a grant from the Medical Research Council during the progress of part of the above work is gratefully acknowledged.



Colour index of rats fed on bread and milk and bled.

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XXI. STUDIES IN ANAEMIA. II.

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(Received January 22nd, 1923.)

COMPARISON OF METHODS OF PRODUCING AN ANAEMIA CHARACTERISED BY A LOW COLOUR INDEX.

MOST investigators of anaemia, in making observations on such points as basal metabolism, circulation rate and blood reaction, and also on such questions as the influence of iron, etc., on blood regeneration, have confined themselves to acute experimental anaemia produced by bleeding. Such an anaemia is at best a transitory phenomenon, the blood soon being regenerated either from a reserve of blood forming materials or from constituents of the diet. The production of a sub-acute anaemia by bleeding and feeding as described in Part I of this paper [1923] is a process so tedious and laborious that before proceeding further it seemed desirable to search for some easier method of producing a more permanent anaemia, and the expedient of breeding from females which had been kept on a diet poor in iron (bread and milk only) from the age of puberty till ten months was adopted. These rats showed practically no anaemia themselves, their colour index being about 0.52 as against the normal 0.56. Healthy males were used for breeding and the process was looked upon as a case where deficiency of some chemical substance in the mother caused a deficiency of the same substance in more marked degree in her offspring.

This was the course adopted by M. B. Schmidt in 1912. Schmidt [1912] found that if a diet of rice and milk were continued in growing mice they gradually became anaemic and were stunted in their growth. The offspring of such mice were studied through several generations throughout which the feeding was continuously "iron-free." If now iron were given to one of a litter of such stunted anaemic mice it quickly outgrew the others and acquired a high percentage of haemoglobin and a nearly normal blood count.

Attention may also be drawn to the work of Brinckmann [1921], who showed that after feeding young guinea-pigs on iron-poor food anaemia of chlorotic type ensues.

In view of our more intimate knowledge of the rat and his greater range of usefulness in experimental procedure it seemed desirable to repeat the experiments with these animals and to institute a comparison between the

case of this process and the process adopted in Part I of this work for the production of anaemia and to compare the degree of chronicity of the anaemias produced.

Rats were bred from healthy fathers and from mothers which had been kept till ten months old on white bread and whole milk. The results of blood examination of the offspring at various ages are shown in Tables I, II and III. It will be seen that as compared with normal controls and with control rats from the same litters which had green stuff added to their diet they show when young a marked depression of the colour index of the corpuscle and that this shows a tendency to ameliorate naturally as the animals get older. Males recover faster than females.

Table I. *Showing blood condition in normal rats at ages 40 and 60 days.*

For adult rats see Table I, Part I.

Age: 40 days. Average weight: 92 g.				
Sex	Hb %	R.B.C. in millions per mm. ³	Colour index (human = 1)	Average colour index
M.	85	7.70	.55	.57
M.	78	6.33	.62	
M.	80.5	7.36	.55	
F.	85.5	7.87	.54	.56
F.	88	8.00	.55	
F.	93	8.10	.58	
Age: 60 days. Average weight: 104 g.				
M.	95	8.86	.54	.57
M.	85	7.54	.57	
M.	93	7.80	.60	
F.	95	8.74	.54	.57
F.	94	7.73	.61	
F.	89	7.67	.58	

RESULTS.

Young rats bred from mothers which had been kept on a diet of white bread and whole milk from puberty till ten months old showed a marked fall in the colour index of the corpuscle owing to the fact that the haemoglobin content of the blood had fallen while the number of corpuscles had risen. This is a phenomenon to which attention may be called. The anaemia was fairly chronic but tended to ameliorate naturally in time on a diet of white bread and whole milk. Its experimental production was easy compared with the method previously adopted in Part I. Control rats from the same litters fed on the same diet but with green stuff added showed a speedy recovery from the anaemic condition.

DISCUSSION.

Hartwell [1921, 1922] has reported work in which she shows that a diet of white bread and milk is adequate for nursing mother rats and indeed gives the best growth curves for sucklings. Sherman and Muhlfeld [1922] also regard a very similar diet of whole milk powder and ground whole wheat as

Table II. *Showing blood condition at various ages of rats bred from mothers ten months old, mothers and young having been kept on a diet of bread and milk.*

A. Females.

Age in weeks	Hb %	R.B.C. in millions per mm. ³	Colour index (human = 1)	Average colour index
8	68.5	10.60	.33	.33
	68.5	10.22	.34	
	68.5	10.58	.33	
10	73	10.78	.34	.33
	73	11.82	.31	
	73	11.00	.33	
12	78	12.00	.33	.34
	78	11.42	.34	
	85	12.22	.35	
16	93	11.18	.42	.42
	88	10.70	.41	
	81	9.72	.42	
22	100	10.32	.48	.50
	105	10.40	.50	
	101	9.67	.52	
24	100	11.20	.45	.51
	117	11.02	.53	
	112	10.40	.54	
	107	10.52	.51	
	100	9.56	.52	
	110	10.60	.51	

B. Males.

8	76	11.36	.34	.35
	61	9.60	.32	
	63	8.10	.39	
10	73	11.10	.33	.32
	73	11.20	.33	
	73	11.90	.31	
13	102	12.58	.41	.35
	61	9.14	.33	
	78	12.88	.30	
18	95	10.48	.45	.46
	95	10.05	.47	
	98	10.63	.46	
22	110	9.92	.55	.54
	105	9.58	.55	
	102	9.76	.52	

Table III. *Showing blood condition at various ages of control rats from the same litters which had been fed since weaning at four weeks old on green stuff in addition to bread and milk.*

Age in weeks	Hb %	R.B.C. in millions per mm. ³	Colour index (human = 1)	Average colour index
8	100	10.60	.47	.49
	95	9.30	.51	
	100	8.82	.56	
	96	10.34	.46	
	94	9.86	.48	
	91	9.56	.47	
10	104	10.10	.51	.48
	104	11.46	.46	
	107	11.60	.46	
13	102	9.42	.54	.54
	93	8.30	.56	
	100	9.65	.52	

adequate for the growth, reproduction, and successful suckling of the second generation. While these conclusions might be justified from the evidence of growth curves alone, yet the work reported in this paper shows that this cannot be interpreted as meaning that these diets are adequate for blood formation. While it is true that we do not know what the blood condition of Miss Hartwell's rats was like, and it may or may not have been abnormal even though growth was normal, the obvious difference between her rats and these was that her rats were bred of robust mothers, who presumably had given them at birth what these rats lacked and were unable to acquire from the poor diet on which they were fed. It would appear that growth curves alone over a limited period of time are not a sufficient test of the adequacy of a diet. An animal may exhibit a satisfactory growth curve and yet be flabby. Further it may have latent a diathesis which manifests itself as anaemia in its offspring.

CONCLUSION.

A simple method of producing a comparatively chronic anaemia characterised by a low colour index and by a tendency to natural cure is described.

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XXII. RESPIRATORY EXCHANGE IN FRESH-WATER FISH.

PART VI. ON PIKE (*ESOX LUCIUS*)

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(Received February 3rd, 1923.)

PIKE form a small family of soft-rayed fishes pretty generally distributed over the rivers and lakes of Europe, Northern Asia and North America. In the old world one species only—*Esox lucius*—is common. Its eastward range is not known, as far as we are aware, but it extends into Lapland in the north and into central Italy in the south; it appears however to be absent from the Iberian Peninsula. It has an elongate rather compressed body covered with small scales, a long head with long and spatulate snout, and a very large mouth armed with strong teeth in the jaws and bands of smaller teeth in the palate and tongue. The teeth point backwards or can be depressed so as to offer no obstruction to any object entering the gape, but prevent its withdrawal in the opposite direction. The pike is the most voracious of fresh water fishes, and many writers have compared it to the shark with regard to the quantity of food it consumes. It leads rather a sedentary than a roving life, and prefers lakes and the sluggish reaches of rivers to strong currents or agitated waters. The dorsal and anal fins are placed far back in the tail, thus greatly increasing the propelling power of the fish, and though relatively poor swimmers pike are excelled by no other fresh-water fish in rapidity of motion when by a single stroke of the tail, they dart upon their prey or out of reach of danger. They appear to be very hardy and we found they could stand confinement remarkably well.

On account of their sluggish disposition and yet great voracity it seemed of interest to determine their oxygen requirements. The fish used for experiment were about 12 inches in length and varied in weight from 110 to 160 g. When placed in the tank they remained very sluggish during the experimental period and though there were plenty of minnows in the tank, the pike did not exhibit their usual voracity, and specimens killed from time to time showed little or no food in the stomach. Probably they were scared by the necessary handling.

The method adopted was that fully described in former papers of this series [1914, 1 and 1922, 1, 2].

EXPERIMENTAL.

At low temperature. Four fish were taken weighing respectively 150, 110, 100 and 140 g. Total weight 500. Initial temp. of water 5.7°, final temp. 6.1°. Duration of experiment 18 hours 57 minutes. 275 cc. commercial oxygen were added during the experiment.

Total free and combined CO ₂	at beginning	1513.2 cc.	at end	1673.6 cc.	diff.	160.4 cc.
“ oxygen	“	5208.1	“	4963.8	“	244.3
“ nitrogen	“	18975.2	“	18973.6	“	1.6

Error in nitrogen 0.0084 %.

At medium temperature. Five fish, 125, 105, 145, 110 and 155 g. respectively. Total 640 g. Initial temp. of water 11.2°, final temp. 13.7°. Duration of experiment 4 hours 17 minutes. 275 cc. oxygen added during experiment.

Total free and combined CO ₂	initial	2164.5 cc.	final	2297.6 cc.	diff.	133.1 cc.
“ oxygen	“	4362.5	“	4181.6	“	180.9
“ nitrogen	“	15655.9	“	15658.9	“	3.0

Nitrogen error 0.0199 %.

Five fish total weight 595 g. Initial temp. of water 16.6°, final temp. 16.65°. Duration of experiment 4 hours 14 minutes. During the experiment 275 cc. oxygen added.

Total free and combined CO ₂	initial	1874.4 cc.	final	2039.9 cc.	diff.	165.5 cc.
“ oxygen	“	4534.8	“	4341.2	“	193.4
“ nitrogen	“	16267.1	“	16275.0	“	7.9

Nitrogen error 0.048 %.

At high temperature. Five fish, 145, 155, 135, 160 and 110 g. respectively, total 705 g. Initial temp. of water 22.9°, final 22.2°. Duration of experiment 4 hours 8 minutes. 275 cc. oxygen added.

Total free and combined CO ₂	initial	1781.2 cc.	final	2026.4 cc.	diff.	245.2 cc.
“ oxygen	“	4514.3	“	4215.4	“	298.9
“ nitrogen	“	16419.8	“	16398.0	“	21.8

Nitrogen error 0.13 %.

These results are gathered together in the following table.

Table I.

Temperature °C.	Oxygen con- sumed per fish per hour cc.	Oxygen con- sumed per kilo of fish per hour cc.	CO ₂ evolved per fish per hour cc.	CO ₂ evolved per kilo of fish per hour cc.	Respiratory quotient
5.7-6.1	3.06	24.44	2.01	16.08	0.66
11.2-13.7	8.45	65.99	6.22	48.56	0.74
16.6-16.65	9.14	76.79	7.82	65.71	0.86
22.9-22.2	14.46	102.58	11.87	84.18	0.82

In this case again the oxygen consumed per fish is approximately proportional to the rise in temperature, and if the oxygen values are plotted against temperature the curve is nearly a straight line.

The respiratory quotients at all temperatures were approximately normal for carnivorous animals.

Table II. *Oxygen consumption per kilo of fish.*

Temperature °C.	Pike	Gold fish	Eels
5°-6°	24.44	16.07	9.28
about 16°	76.79	50.09	44.40
22°	102.58	83.81	61.29

On comparing the oxygen consumption of pike with that of eels and gold fish, it will be seen from Table II that at the various temperatures pike require more oxygen than gold fish in the ratio of about 3 : 2, and that gold fish in this respect occupy a position midway between pike and eels.

Quantity of oxygen in the water at the asphyxial point for pike.

It seemed of interest to determine the behaviour of pike under reduced oxygen tension and measure the quantity of oxygen in the water at the asphyxial point.

Experiments were carried out at a medium temperature and the apparatus and method fully described in Part II of this series of papers [1914, 2] were used. No experiments were made at low temperatures as it did not seem likely that it would be possible to reduce the oxygen in the water to the asphyxial tension within a reasonable time, and the fish at our disposal were too large for the small apparatus used for gold fish in Part IV [1922, 2].

A constant stream of nitrogen was passed through the experimental bottle during the night to reduce the oxygen tension in the water and the fish were introduced the following morning.

Four fish weighing respectively (1) 90, (2) 150, (3) 152 and (4) 120 g. were selected. Two fish were placed in the bottle at 10.58 a.m., the temperature of the water being 15.3°. Both fish remained inactive at first and the respiration rate was about 77 per minute. Minute bubbles of gas were seen to rise occasionally from the smaller fish, but no sign of distress was apparent in either and movement was only occasional. At 11.40 a.m. the other two fish (3 and 4) were introduced, and nitrogen was circulated through the water by the pumping apparatus. The bubbling of fresh nitrogen through the water sampling tube and the pumping through the spraying tube were carried on simultaneously.

At 12.45 p.m. one of the fish began to show marked activity and was seen to rise to the surface. All the fish then became more active and rose to the surface frequently. At 1.20 p.m. one of the fish gave a violent leap out of the water, which was repeated a little afterwards. The frequency of respiration of one of the fish at 1.45 p.m. was 60-70 per minute. Increased activity, accompanied by vigorous leaps out of the water, was observed until about 2.45 p.m., after which the fish again became sluggish and their movements more spasmodic with longer intervals of rest. At 3.45 p.m. one fish gave a violent leap and then turned over. After some spasmodic movements the fish settled on its back, with occasional convulsive gasps. The other fish showed signs of getting into the same condition. At this point a sample of water was withdrawn for analysis, and the current of nitrogen then replaced by a rapid current of oxygen. At 4.45 p.m. the fish which had been most affected again assumed a normal position. On removal to the outdoor tank the fish

appeared to have quite recovered and continued perfectly healthy for many weeks, and were ultimately killed for other purposes.

Analysis of sample of water, the temperature of which when collected was 14.1°.

The gas dissolved in the water sample (45.995 cc. of water) was pumped out in the usual way. It exerted a pressure of 10.225 mm. Hg at 11.64° and constant volume 36.62 cc.; after shaking with 40 % KOH it exerted a pressure of 1.83 mm. Hg at 12.08°; and after shaking with alkaline pyrogallol, a pressure of 1.80 mm. Hg at 12.60°. The volumes of CO₂ (free and combined), oxygen, and nitrogen dissolved per litre of water were therefore 84.38 cc., 0.334 cc., and 18.025 cc. respectively, measured at s.t.p. The coefficient of absorption of O₂ in water at 14.1° is 0.03479.

Hence the tension of dissolved oxygen was $\frac{0.334}{10 \times 0.03479} = 0.96$ % of an atmosphere, i.e. 7.30 mm. Hg.

Evidently pike, like gold fish and eels, can exist at tensions considerably below normal without harm.

Limits of temperature within which pike can live.

In order to determine the highest temperature at which pike can live, a fish *A* weighing 150 g. and another *B* weighing 107 g. were placed in a tub of cold water at 11°, and the temperature was very gradually raised to 18–19° by careful addition of warm water. The water was kept fully oxygenated by spraying oxygen through by means of a circular lead coil perforated with fine holes. The temperature was very gradually raised, but no sign of discomfort was observed until the temperature had reached 27°. The rates of respiration noted at various temperatures were as follows:

Temperature	11°	18°	22°	26°	27°	29°
Respiration rate	56	47	45	40	55	63

At 27° the respirations were markedly deeper and the fish became more active. Movements became slightly convulsive in the neighbourhood of 30°, and at this temperature both fish turned over. The fish were then immediately removed to cold water, and on return to the outside tank quickly revived and appeared to have suffered no harm. They were alive and healthy many weeks after the experiment.

In our experience pike are remarkably hardy in confinement, and consequently very suitable for experiments such as have been described, and, as we hope to show later, for experiments on the effects of toxic substances.

We take this opportunity of expressing our thanks to Mr A. R. Peart of the Berkshire Trout Farm, Hungerford, for his kindness in procuring suitable fish and for much most valuable advice and information.

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XXIII. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM* *COLPODA*.

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(Received February 6th, 1923.)

INTRODUCTION.

THAT protozoa are important factors in soil economy is becoming increasingly evident since there is no question that large numbers of them pass through the various phases of their life history in the soil, and during their life influence the other members of the population. In a previous paper [Cutler, Crump and Sandon, 1922] it has been shown that the numbers of active protozoa of various species change rapidly from day to day without any obvious reference to gross environmental changes. Thus in the course of a year's daily counts in field soil, no correlation could be traced between the fluctuations in numbers of six species of protozoa and the rainfall, temperature or moisture content, and moreover the species appeared in the main to be living independently of one another.

It therefore appears that these variations in numbers are the expression of the animal's life cycle, or else that there is some obscure factor or combination of factors at work in the soil limiting activity. This second alternative is of course possible. If, however, the first suggestion is the true one growth in pure cultures should follow the same general course as obtains in the soil. For the two species tested, *Colpidium colpoda*, a ciliate, and *Oicomonas termo* (Ehren.), a flagellate, this is the case; for after the first few days of incubation, during which the culture is becoming populated, irregular variations in numbers occur, which are in every way comparable to the soil fluctuations.

The experiments detailed below were undertaken with a view to elucidating these changes, and to finding what are the factors in liquid culture determining the variations in reproductive rate and death.

From the point of view of soil biology such information is essential, but it is of added importance in respect of the various theories that have been advanced in explanation of growth. This has been dealt with extensively for the metazoa, metaphyta, and bacteria, but little is known regarding protozoa. Recently, however, Robertson [1921] has published experiments which he believes demonstrate that in certain ciliates growth is auto-catalytic

in character similar to that which has been recorded for higher organisms. Further research is necessary either to prove or disprove this view but the evidence adduced is not convincing.

The following account deals only with the earlier stages of growth in mass culture, later stages having been dealt with in detail by various authors.

In a future communication it is hoped to describe experiments on single cells isolated into drops of culture media of varying volumes.

METHODS.

The first essential in work of this kind is a rigorous standardisation of the cultural methods, so that any experiment can be repeated, or carried out in duplicate, with a reasonable expectation of obtaining consistent results, the organisms themselves being as far as possible the only variable factors.

Two organisms, whose source will be considered later, have been used throughout these experiments, *Oicomonas termo* and *Colpidium colpoda*. The method of estimating growth has been to count the number of animals in representative samples of the culture fluid in a counting chamber. The most convenient type for counting organisms of this size is the Cropper ruling, where an area of 2.5 sq. mm. is divided up into 625 squares of 0.04 sq. mm. area, the whole chamber being 0.1 mm. deep. The experimental error of the counts depends upon the number of animals counted, provided that the organisms are uniformly suspended in the fluid; then, if x is the number counted, the actual number present will lie between the limits $x \pm \sqrt{x}$ [Student, 1907]. In every case the number per cc. is given¹. The animals are killed before counting by adding a small drop of lugol to the counting chamber. The experiments have all been carried out in the following way: 100 cc. flasks of quartz or Jena glass containing a known amount of medium (10 or 20 cc.) are used for the cultures. The number of organisms in the parent culture is counted and a quantity of liquid varying from 0.1 to 1.0 cc., according to the strength of inoculum required, is pipetted over into a new flask. In every case where the numbers of organisms in the sub-cultures have been counted immediately after inoculation the expected number has been found. The cultures are incubated at 19°.

Preliminary work suggested the following experimental conditions, variation in which would introduce errors:

1. Composition of medium.
2. Glass ware.
3. Aeration.
4. Temperature of incubation.
5. Strain of organism.
6. Food supply.

¹ The reproductive rate for any time is calculated from the formula: $\frac{\log B - \log A}{\log 2}$ where A = no. at beginning of time and B at the end.

1. *Composition of medium.* For comparative work organic infusions are wholly unsuitable, as it is impossible to reproduce them exactly. For this reason hay infusion has been discarded, although both *Oicomonas* and *Colpidium* thrive in it, and a synthetic medium has been used instead. In the first experiments on *Colpidium* Peters's "ammonium glycerophosphate medium" was used [Peters, 1921], or else one in which 0.06 % ammonium phosphate and 0.25 % saccharose were substituted for the glycerophosphate; the later experiments were carried out in a variation of Peters's glucose and lactate medium which contains the following compounds:

Na ₂ HPO ₄	0.001 %	MgSO ₄ , 7H ₂ O	0.0001 %
Ammonium lactate	0.01		CaCl ₂	0.002
KCl	0.03	Glucose	0.04
NH ₄ Cl	0.03			

Colpidium has flourished on this medium for more than five months. According to Peters the optimum growth is obtained in this ciliate when the reaction of the medium is initially p_H 7.0 to 7.4. The medium has therefore always been adjusted to an initial reaction of approximately p_H 7.2, but satisfactory growth has been obtained at values outside these. Thus cultures starting at p_H 7.6 and 6.8 have shown apparently normal growth, and judging by the fact that Dale [1913] found the limiting values for growth in *Paramoecium caudatum* to be p_H 5.0 and 9.0 the range is probably very much wider.

Also a series of experiments made by S. M. Nasir in this laboratory showed that both ciliates and flagellates could live and reproduce in artificial media of p_H 3.9.

Peters's suggestion of adding phenol red in minute quantities to the cultures has also been followed, so that changes in acidity and alkalinity during the life of the culture can be noted; the routine procedure is to add two drops of the indicator to 20 cc. of medium. The final modification of the medium very gradually becomes alkaline as growth proceeds.

2. *Glass ware.* At an early stage in the experiments it was found that certain flasks, notably those made of Bohemian glass, had a bad effect on the growth of the organisms; in some cases no growth occurred at all. All the recorded experiments have been carried out in Jena glass or quartz flasks which give uniform results and do not affect the p_H value of the medium even after repeated sterilisation in the autoclave.

3. *Aeration.* Differences in aeration were found to have a marked effect on cultures of *Oicomonas*. Three series of cultures, each containing three parallels, were put up in the ordinary way: the first series were plugged with cotton wool, the second with corks through which a piece of bent glass tubing ran, and the third were attached to a filter pump so that a steady stream of air was drawn through them. In the second and third series the tubes leading to the outside air were so bent that no contamination of the flasks could occur. The results of this experiment are shown in Fig. 1. It is obvious that extreme aeration has a very disturbing effect on the growth of the cultures, and that

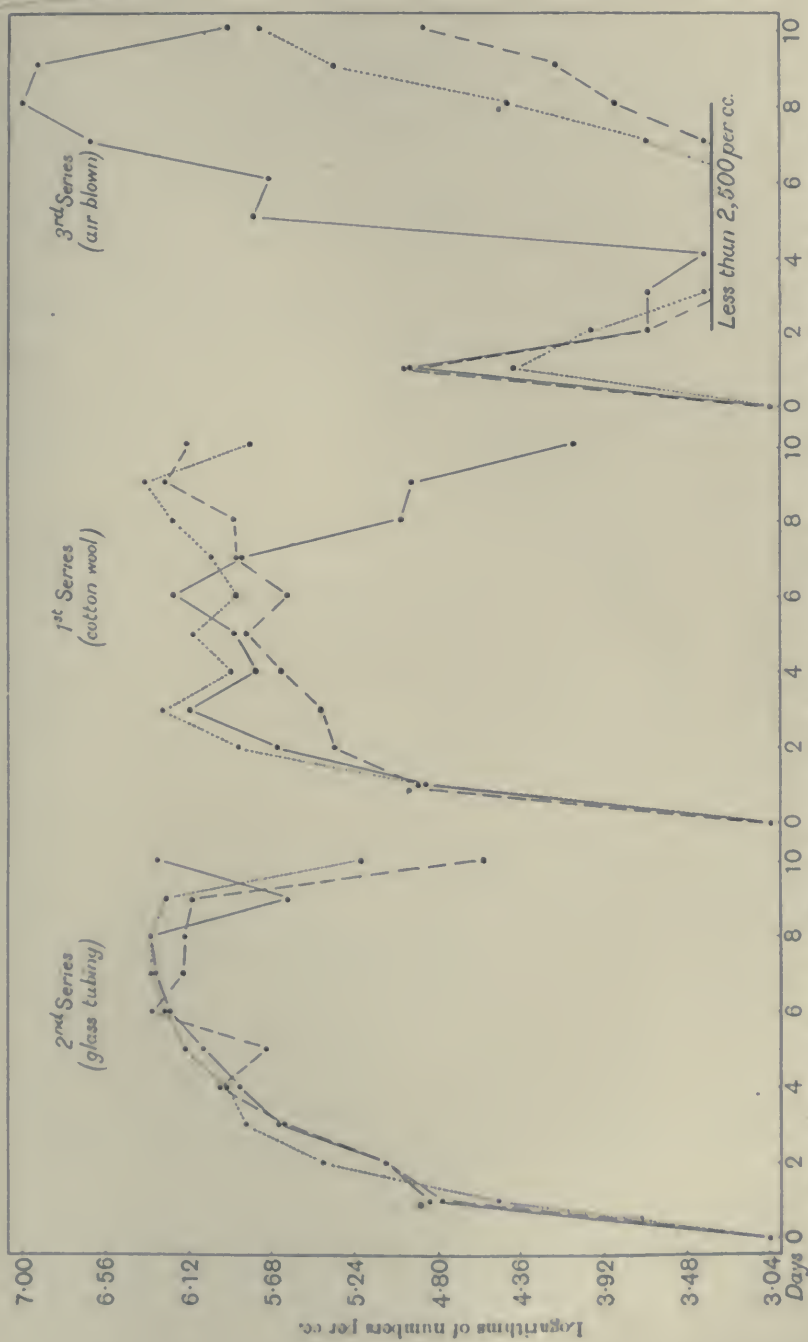


Fig. 1 Curves showing the effect of aeration on the reproduction of *Oicomonas*. The asterisks mark the points where conjugation forms were first observed.

better parallels are obtained when the aeration is absolutely constant (2nd series) than where there is even a slight variation (1st series).

4. *Temperature of incubation.* The incubation temperature throughout all the experiments has been as far as possible 19° ; the effect of changing temperature is clearly shown from the results of the following experiments where two sets of flasks were incubated at 19° and 24° respectively (Fig. 2). Although

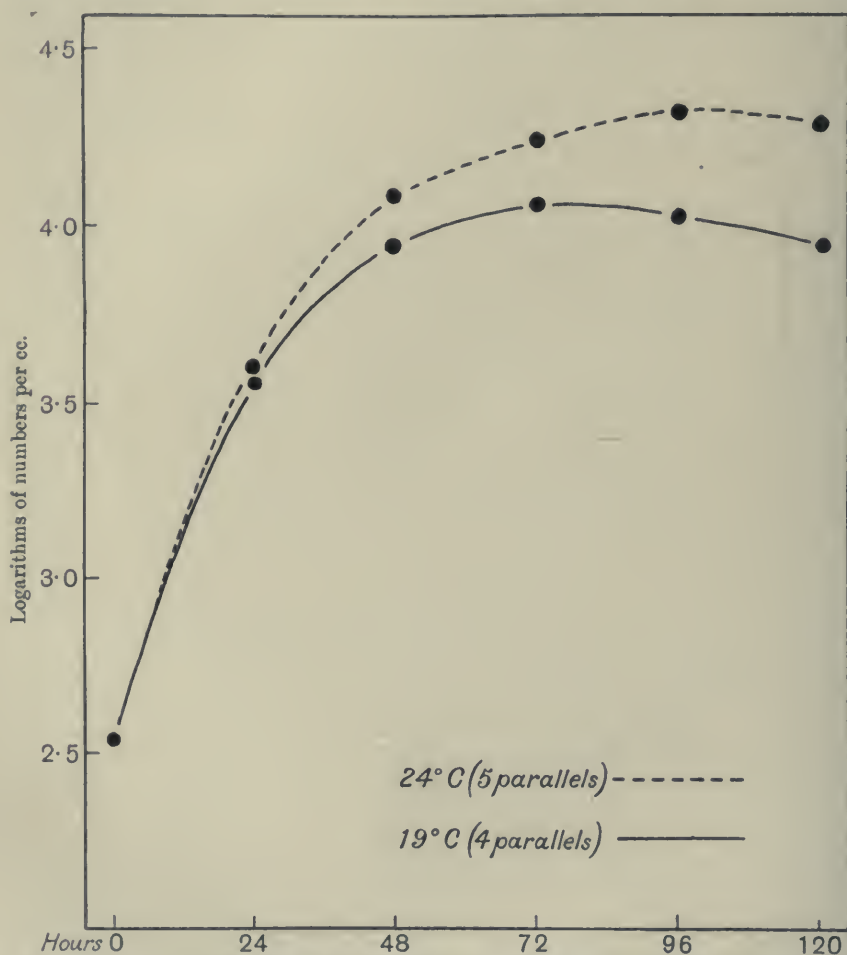


Fig. 2. Curves demonstrating the effect of temperature on the reproduction of *Colpidium*.

24° would appear to encourage more rapid, and even greater, reproduction in *Colpidium*, it is possible to maintain better experimental conditions at the lower temperature, as it approximates more nearly to that of the room and the shock of transferring the flasks from the incubator to the bench is very much less. In the experiment involving three hourly counts (cf. p. 180) a bath kept at 19° was arranged on the bench by the microscope and each flask was placed in it while the counts were made; McKendrick and Pai [1911] found this

a very necessary precaution where working with bacteria at incubator temperatures of 37°; at the lower temperatures it is probably an unnecessary refinement.

5. *Strain of organism.* The variation introduced by the strain of organism is probably considerable, since different strains show consistent differences in many characters. Thus, known differences in strain include such characters as size, rate of multiplication and frequency of conjugation [Jennings, 1920], resistance to heat [Hutchison, 1913; Jollos, 1913], the readiness with which conjugation occurs [Hopkins, 1922] and resistance to poisons [Jollos, 1913]. All the experiments on *Oicomonas* have been carried out on a single strain, the original culture being obtained from soil from Barnfield dunged plot; it is believed that the cultures came from a single cyst, though, owing to the small size of this organism, it is difficult to assert this positively. The cultures of *Oicomonas* are contaminated with at least three species of bacteria. Conjugation is of very frequent occurrence in *Oicomonas termo* thus making it from the point of view of the present work an unsatisfactory species to use. After the first appearance of the large conjugation forms (marked in each figure) the counts are subject to a considerable source of error; up to this point, however, they are accurate within the limits of experimental error. In the case of *Colpidium* all the experiments have been carried out on the same strain. The original stocks were given to us by Dr Peters and each had been derived in the first instance from a single cell. All our stocks of this ciliate are contaminated by a very small, stout, gram positive bacterium, which is present only in small numbers. Conjugation has never been seen in any of the cultures although they have been under close observation for more than six months, and only a very few isolated cysts have been found during that time.

6. *Food supply.* In the growth of a culture two factors are involved: the growth in size of the individuals, and the increase in their numbers, an increase, brought about in the cases under consideration, by binary fission. To attack the problem of growth is therefore by no means simple. The actual increase in size in any one animal depends in great part on the food supply¹, at least in young cultures; for this reason the most profitable method has seemed to be to provide a surplus of acceptable food and then to treat the increase of numbers as indicative of the actual increase in protoplasm. The *Colpidium* cultures have been fed either at inoculation or after 24 hours' growth with a pure culture of *Sarcina lutea* in sufficient quantity to ensure that a supply of it is present throughout the experiment. In the case of *Oicomonas* the contaminating bacteria provide abundant food. Even with these precautions the increase in the amount of living protoplasm in a culture can be found only by regarding both reproductive rate and average size of the animals. At present, however, our attention is confined to rate of reproduction.

¹ A culture of colpidia in which the mean length of 30 animals was 15 μ and the mean breadth 5 μ and in which there was a scarcity of bacteria was fed with *sarcina*; on the following day the mean dimensions for 30 animals were 20 μ \times 11 μ .

RESULTS.

The general course of a curve plotted from counts made at 12 or 24 hourly intervals on a mass culture of *Colpidium* is roughly sigmoid for a period varying from 2-6 days. There is then a fall in the numbers, and after this first maximum the curve becomes wholly irregular approximating closely to the type of curve obtained by counting protozoa in soil. As conjugation has not been taking place in any of the cultures it is obvious that death is the cause of the drops in the numbers. Experiment has shown, however, that death occurs in the earlier stages of the culture as well, and that the initial smoothly rising part of the curve can be resolved into an irregularly rising line by making counts at shorter intervals of time¹. For this purpose three hourly counts were made extending over a period of 102 hours on eight cultures, four being made from a 24 hour old parent and four from one of 96 hours. In Figs. 3 and 4 curves derived from a typical culture from each series are shown. In each case the curve obtained from the 12 hourly observations shows a steady rise, while the 3 hourly line rises and falls irregularly. If only the 24 hourly observations are plotted, the curves are again completely changed, as in each case 2 maxima, which occurred at night, escape notice. It is not surprising that such being the case, it has not proved possible to apply the commonly accepted autocatalytic formula² to any of the colpidium curves.

Death following inoculation.

A further complication, which must be recognised, before any general theory of growth and reproduction can be made, lies in the fact that death also occurs under certain conditions immediately after inoculation. Such is the case in cultures made from old parents. This is best illustrated by reference to the following experiments on *Colpidium* and *Oicomonas* (Figs. 5 and 6). In these, sub-cultures were made from the same stock culture at intervals of 12 or 24 hours and the numbers of protozoa in the sub-culture were counted after 12 and 24 hours and afterwards at 24 hourly intervals. During the first 12 hours after inoculation the sub-cultures from the older parents show a decrease in numbers; this, however, would pass unnoticed if the counts at 12 hours were omitted and the curve would then show the lag which is commonly found in bacterial cultures. Thus McKendrick and Pai [1911] with bacteria counted at half-hourly intervals found a lag when their cultures were derived from old parents (14 days as against 1-3 hours), which did not appear in cultures from young parents. Penfold [1914] found that the older the parent culture in the case of *B. coli* within limits, the longer is the lag period in the sub-cultures. In the protozoa Calkins [1919] has found in cultures of *Uroleptus* derived from single ex-conjugants, that old age in the parents leads to a series of low vitality, as evidenced by the duration of life and by reproductive

¹ In the case of certain bacteria Wilson [1922] finds that there is a normal death rate even during the period when the maximum rate of growth is proceeding.

² $\log \frac{x}{a-x} = k(t-t_1)$.

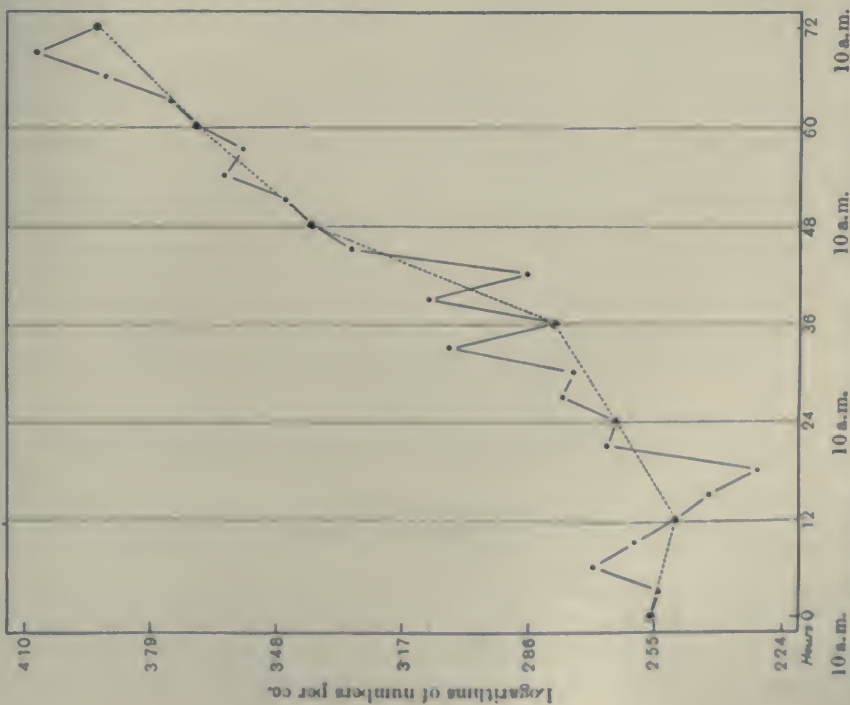


Fig. 3. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 96 hours.

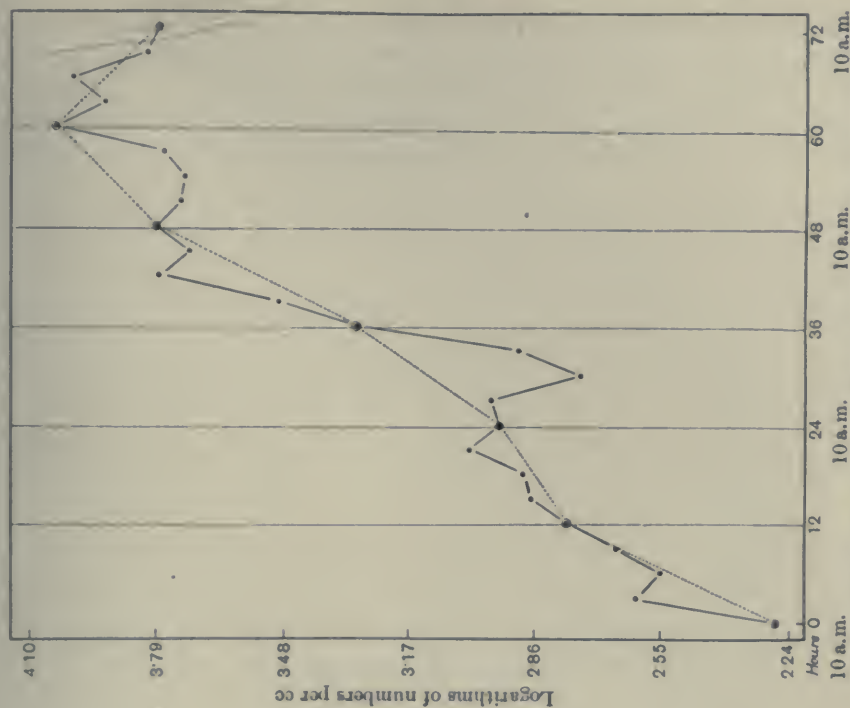


Fig. 4. Curve showing the reproductive rate of *Colpidia* from counts made at three hourly intervals. The broken line gives the curve obtained if 12 hourly counts had been made. The age of the parent was 24 hours.

ability, while young parents produce series of relatively high vitality. Robertson [1921] also adduces a certain amount of evidence bearing on this point. Starting from single individuals of *Enchelys* he finds that on an average

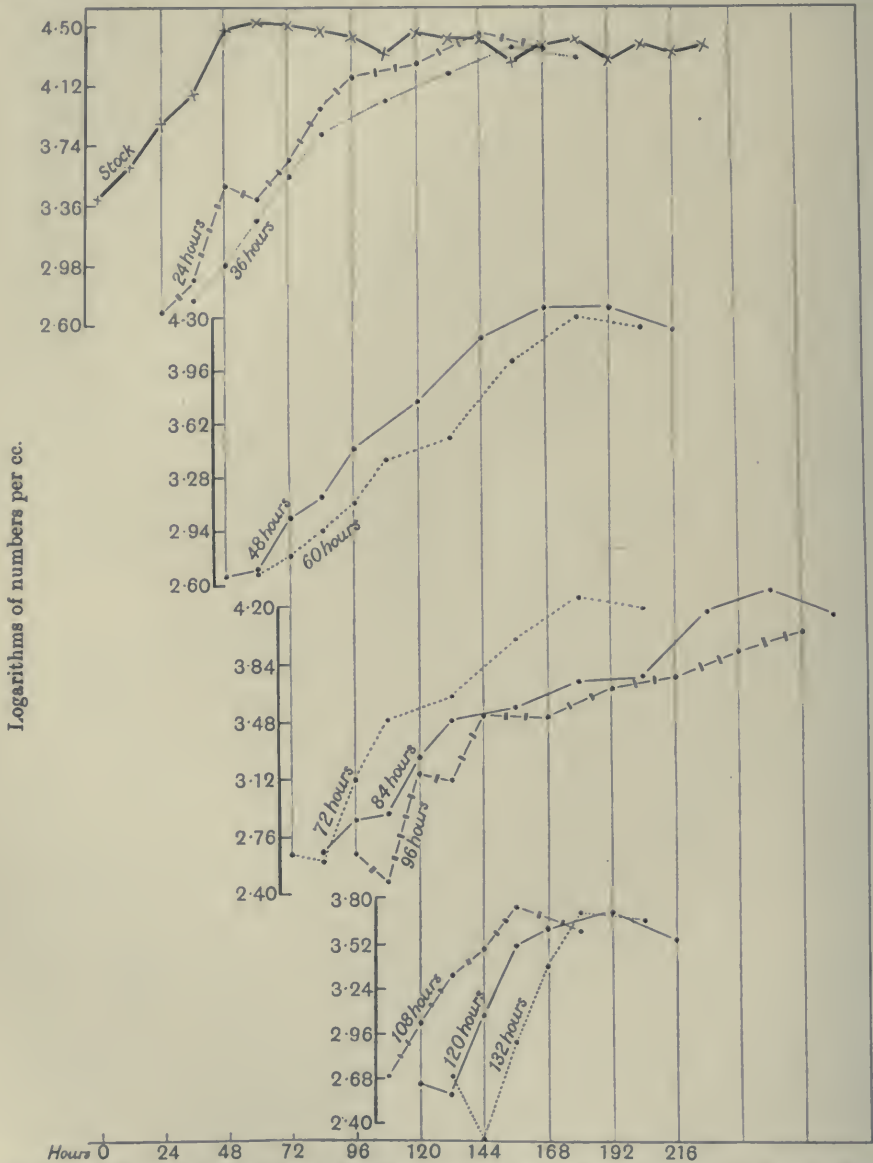


Fig. 5. Curves showing in *Colpidium* death following inoculation according to the age of the parent culture, which is written over each curve.

a one day old parent produces 38.4 in 24 hours, a two day parent 5.9, a three day parent 2.6 and a four day parent 2.0. In the case of the one day old parent, where he obtains the startling reproductive rate of 5.26 in 24 hours, he cites 27 cases in which the number of progeny produced vary from 11-105.

It is possible that in the case of the initial death the transfer of cells from a stale medium to a fresh one may involve too violent a change in the physical and chemical conditions, and that death is due to these causes. Certain facts, however, militate against such a suggestion. For instance, in the series of experiments with *Colpidium* (see Fig. 5) there is death on inoculation in both

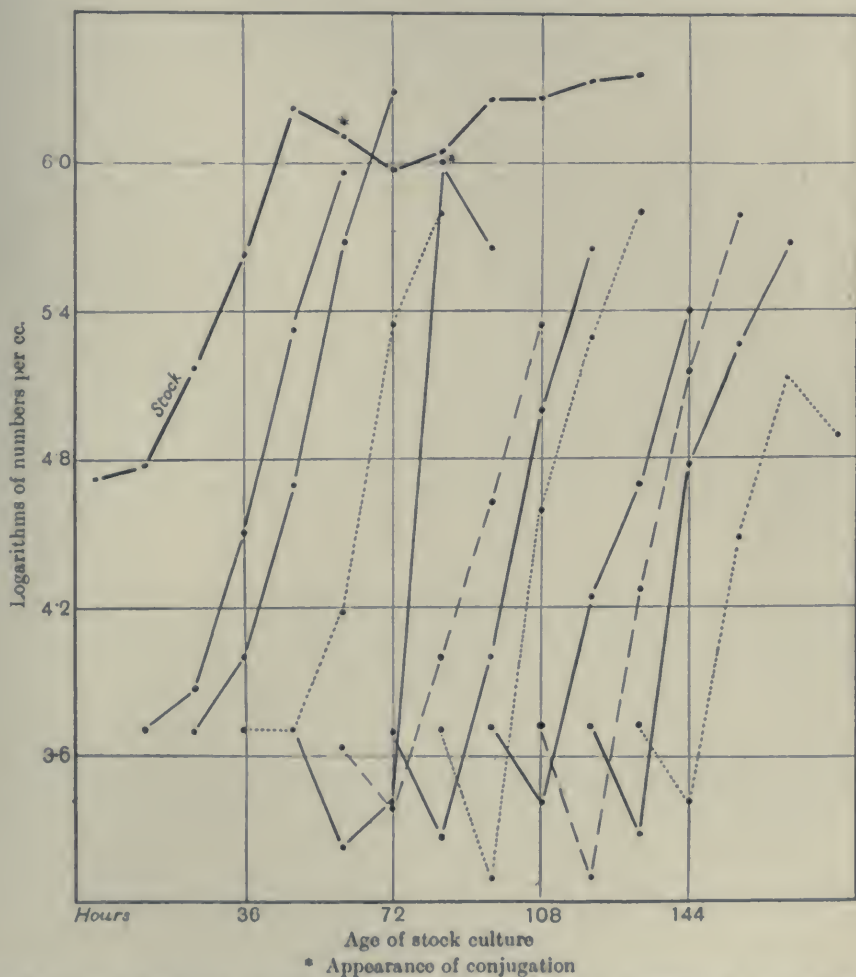


Fig. 6. Curve showing in *Oicomonas* death following inoculation according to the age of the parent culture. The asterisks mark the points where conjugation forms were first seen.

sub-cultures made from the 96 hour parent, but not in those made at 108 hours. It is interesting to notice that the stock culture decreased in numbers between 96 and 108 hours, but between 108 and 120 rose suddenly, which suggests that the initial rise in numbers in the 108 hour sub-culture was due to something inherent in the animals, which the change in medium is powerless to stop.

A further attempt has been made to clear up this point by sub-culturing

from the stock at various ages into its own fluid which is previously filtered. In every case there were very much lower numbers in the filtered liquid than in the fresh medium, but the results are vitiated by the fact that fresh medium when filtered gives lower numbers than control unfiltered medium, indicating that filtration changes the medium too much to permit of any deductions being drawn from such experiments.

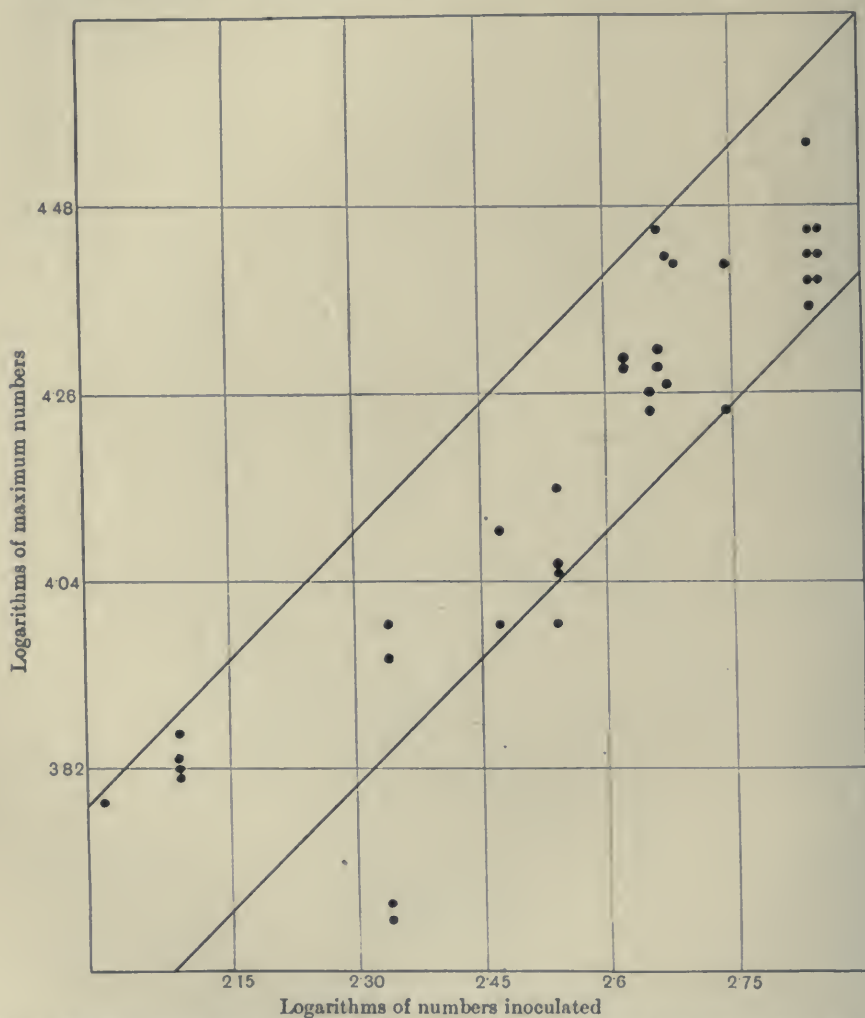


Fig. 7. Figure demonstrating the relation between the numbers of *Colpidium* inoculated and the maxima attained.

Constant reproductive rates.

Old age in the parent culture also influences the later growth of the sub-cultures (Fig. 5), the maximum numbers in such cultures tending to remain very much lower than they are in those from young parents. These lower

numbers which are obtained from old parents are undoubtedly in part due to the impaired vitality to which Calkins draws attention, but the initial death, by reducing the number from which the organisms have to rise, will also tend to keep the final numbers low. The number of organisms per cc. with which a culture starts, appears to influence it throughout its existence to a marked extent. In Fig. 7 the logs. of the numbers of animals inoculated are plotted against the logs. of the maximum numbers attained in the same culture. Each point therefore represents the average number of times that each animal has divided. In 91 % cases the points fall between the lines which include from 5 to 6 divisions.

The highest number ever reached by any of these cultures was about 35,000 animals per cc., but in other experiments there have been as many as 64,000 colpidia per cc.; therefore the comparatively low numbers reached in some cases cannot be in any way due to the environment itself, as is evident by the fact that young rapidly divided animals inoculated into such media are not killed as they would have been had the medium been toxic.

There is a great degree of variation in the size of the inoculum (100-700 per cc.), the age of the parent culture (24-48 hours) and the time taken in reaching the maximum number (48-168 hours) in the cultures represented. It is further of interest that the reproductive rate during the first 24 hours is very varied and apparently bears no relation to the final numbers attained.

CONCLUSIONS.

When the work now in progress on single cell cultures has been extended it is hoped to discuss in detail the experiments described above with especial reference to their bearing on the results already obtained from the investigation of protozoa living in the soil.

For the moment it is sufficient to point out how important it is that the experimental methods employed should be standardised if comparative results are to be obtained. To the physiologist this is self evident, but, judging by the papers already published, it has not been sufficiently appreciated by students of micro-biology. Also, to gain an insight into the life cycle of any species of protozoa, it is necessary to make observations, not only over a long period of time, but at frequent intervals. The three hourly count experiment recorded above shows how much, that is probably highly significant, is lost if the cultures are left unattended for even 24 hours. Finally it cannot be too strongly urged that in further work of this nature at least three, and preferably five, parallels to each experiment should be put up.

As regards the actual results obtained it would be premature to attempt an explanation of such phenomena as death following inoculation according to the age of the parent culture, and the constant reproductive rate of certain strains of *Colpidia*.

If in bacterial cultures a connection between the age of the parent and

death following inoculation also obtains it has an important bearing on the method sometimes used of differentiating between strains according to the time taken to ferment certain sugars.

In conclusion we wish to express our thanks to Dr Peters for providing us with the cultures of *Colpidium*, and to Mlle. Perey and Mr Sandon for valuable help during the short period counts.

SUMMARY.

1. Methods are given by which it has been found possible to obtain comparable results when studying the reproductive rates of certain protozoa in mass cultures.

2. It is shown that within a relatively short period after inoculation, under certain conditions, a varying proportion of the organisms die; and that this is correlated with the age of the culture from which the inoculation was made.

3. By means of three hourly counts it was found that death occurs even during the period of maximum reproduction.

4. Evidence is supplied that in certain strains of *Colpidium* the rate of reproduction from inoculation to the maximum numbers attained is constant.

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XXIV. INFLUENCE OF A MILK DIET ON THE SKELETON.

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RICKETS is developed principally at the age when milk forms the staple diet. Most authors (Aron [1908], Schabad [1910], Dibbelt [1910], Korenchevsky [1922, 3]) draw attention to the fact that a child fed on a milk diet is liable to suffer from calcium starvation, which is an important factor in the development of rickets. Pfeiffer [1885] found that the milk of mothers of rickety children might be less rich in phosphorus. According to the investigations of McCollum and co-workers [1921, 1922] this deficiency, in conjunction with the deficiency of a fat-soluble factor, may be one of the causes of rickets. Daniels and Loughlin's experiments [1920], if confirmed by experiments on a greater number of animals, might explain the cause of the decreased content of salts in the milk: these two authors found that pasteurisation and prolonged heating caused the loss of calcium salts from milk. Rats fed on such milk did not grow, and even perished from exhaustion. The addition of calcium salts (especially tricalcium phosphate or calcium glycono-phosphate) was followed by a resumption of normal growth. This result could not be obtained by adding vitamin A or B to the milk.

The work of Mellanby [1919, 1920, 1921] and Korenchevsky [1921, 1922, 1, 2, 3; 1923, 1, 2] showed the importance of a deficiency only of a fat-soluble factor in the production of rickets, and Korenchevsky's investigations showed the importance of the simultaneous deficiency of the fat-soluble factor *plus* calcium. In the present work, "fat-soluble factor" is applied to a factor or factors connected chiefly with animal fats, and conducing to the normal metabolism of calcium in the organism, to growth, nutrition, and appetite in animals. We consider "fat-soluble factor" a suitable term in view of the fact that the existence of a fourth, anti-rachitic, vitamin, apart from vitamin A [McCollum and co-workers, 1921, 1922], has not yet been fully proved. The existence, in milk, of a fat-soluble factor possessing anti-rachitic properties raises the question of its possible deficiency in some milk, and therefore, of the possibility of a milk diet producing rickets in consequence of this. The investigations of Hughes, Fitch and Cave [1921], Drummond, Coward and Watson [1921], Dutcher [1921], Kennedy and Dutcher [1922] showed that the content of vitamin A in cow's milk or in butter made from it may fluctuate

very greatly, principally in dependence on the vitamin content in the food of the cow.

In making butter, part of the vitamin A contained in the milk is inactivated [Drummond, Coward and Watson, 1921]. In some samples of butter these authors found a surprisingly small content of vitamin A. On the other hand, Hopkins [1912, 1920] found that the addition of 2 cc. of fresh milk to the food deprived of vitamins was enough to maintain the normal growth and health of rats, that is to say, some kinds of milk are really very rich in vitamins. The factor acting destructively on vitamin A is oxidation, especially on heating, but not heating alone [Hopkins, 1920; Drummond and Coward, 1920; Zilva, 1920; Mellanby, 1921; McCollum and co-workers, 1922].

In the present investigation we shall not quote all the literature on the influence of fresh, heated or desiccated milk on the growth, nutrition and reproduction of animals. Such literature has been collected and properly compared in the works of Mattill and Conclin [1920], and Daniels and Loughlin [1920]. In the summary of their investigation Mattill and Conclin give a clear idea of the present position regarding this question. In their experiments, young rats after weaning were placed on various rations, consisting primarily of cow's milk, fresh and desiccated. On fresh milk rats made good initial growth, but beginning between the 50th and 100th day of life, especially in the females, there was a decided retardation. There was no reproduction. The addition of yeast filtrate temporarily increased the rate of growth, and one female became pregnant, but the litter was eaten. Addition of wheat embryo also increased the rate of growth, with no further effect thus far. When milk was supplemented with iron citrate, growth was much more satisfactory, but reproduction was not successful. The growth failure was in part the result of the dilute form of the food, for on dry milk rats made much better growth, and their average food consumption in milk solids was considerably greater than that of those fed on fresh milk. The females, however, remained somewhat below normal after 75 days, and again there was no reproduction. Neither substitution of dry skim milk to which butter fat was added equivalent to that in dry whole milk, nor the addition of 10 % of butter fat to dry whole milk, was successful.

On a ration containing 55 % of dry whole milk, 40 % of starch, and 5 % of butter fat, both male and female rats made practically normal growth, and the females bore young, but did not rear them. A similar ration has been used by other investigators, who secured not only satisfactory growth, but also reproduction, while others again have, like ourselves, not had success with whole dry milk alone. Dilution of dry milk by 25 % lactose resulted in poor growth and no reproduction. The testes were, with one exception, of normal weight, and contained motile spermatozoa. The ovaries, on the other hand, were much under normal weight, even 50 % and more. In Mattill and Conclin's opinion, possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth

and reproduction, especially in the female, and it may contain substances that are inhibitory to the growth of an animal in the third or mature growth cycle. A ration containing dry milk and 1 % of yeast is the only one on which normal growth and partially successful reproduction were obtained, and the growth of rats on this ration, and on one containing 5 % of yeast, is thus far practically normal. Whether yeast supplies something unique remains to be seen.

As far as we know, there have been only three investigations of the influence of a milk diet on the skeleton. Bolle [see Bartenstein, 1905] found that milk, either fresh or boiled for five minutes, on which guinea-pigs were fed almost exclusively, produced no skeletal changes in the latter. Milk boiled for 10 or 20 minutes produced osteoporosis, fragility of the bones and a partial separation of epiphyses in the skeleton of guinea-pigs. The skeletons were not examined either microscopically or chemically.

Keller [see Bartenstein, 1905] obtained negative results on repeating Bolle's experiments on mice and dogs.

Bartenstein [1905], on the contrary, obtained, on the whole, the same results as Bolle. In his experiments, the daily ration of the guinea-pigs consisted of milk *ad libitum*, with the addition of cream (up to 8-12 % fat), 2 g. of hay and 1 g. of wheat bran. Unfortunately, for the purpose of histological examination the bones were completely decalcified in H_2SO_4 , and therefore the question of the presence of rickets could not be solved. The author considers the resultant histological picture analogous to the osteotabes infantum of Ziegler [1901].

Thus, up to the present time there have been no investigations, carried on in accordance with modern technical requirements, for the purpose of determining the influence of a milk diet on the skeleton. The object of the present investigation was to ascertain the influence on the skeleton of rats of diets consisting of various doses of fresh, heated and oxidised milk. Oxidation was attempted for the purpose of diminishing, or destroying, its anti-rachitic properties in order to produce rickets in the rats by such a diet. In all there were three series of experiments. The object of the first series was to study the effects of large doses of milk; that of the second series—the effects of medium doses, and of the third series—of small doses. The histological and chemical methods of investigating the skeleton were the same as those used in previous investigations [Korenchevsky, 1921, 1922].

FIRST SERIES OF EXPERIMENTS. LARGE DOSES OF MILK.

Seven experiments were conducted on 51 rats, milk being given *ad libitum*. 3 % of cane sugar was added to the milk, in order to increase its calorie content. The milk was supplied by a large London firm, and was a mixture of milk from different cows. The experiment lasted from December to 10th March, i.e. "winter" milk was used.

The milk supplied was divided into three parts: one was left fresh (FM), the second was heated in a bath of boiling water in a flask for seven hours (HM); the third (OM) was aerated by a brisk stream of air and at the same time heated in a bath of boiling water for seven hours, for the purpose of oxidising the fat-soluble factor. Before being passed through the milk, the air was heated in a coil, likewise immersed in boiling water. Owing to this the temperature of the milk during oxidation averaged $98^{\circ}\cdot 8$ C. Milk thus oxidised was used in experiments Nos. 36-42 of this series.

The calcium content of the milk when boiled for ten minutes, heated in a bath of boiling water, with or without aeration, was hardly altered, as shown by our analysis of nine samples of milk. The average figures were as follows:

(1) Fresh milk	0.122 % Ca
(2) Boiled for ten minutes	0.123 % Ca
(3) Heated for seven hours in a bath of boiling water	0.121 % Ca
(4) Oxidised and heated for seven hours	0.113 % Ca

That is to say, only the oxidised milk showed a slight decrease (about 7 %) below the normal in its calcium content. The milk itself became thicker on aeration: this was not due to the evaporation of water, as a reflux condenser was placed over the flask during oxidation for the purpose of preventing evaporation. In order to make sure that the resulting skeletal changes on a milk diet were due solely to the changes in the amount of the fat-soluble factor, and not to that of vitamins B and C, each rat received daily, over and above milk with 3 % sugar, 3 g. of BC I paste, composed of the following:

Paste BC I:	Starch	...	57 g.
	Yeast	...	11.4 g.
	Orange juice	...	13.6 cc.
	Oxidised milk	...	18 cc.

For control purposes, during the last third or half of the period of milk diet, some of the rats were given, in addition to paste BC I, a daily additional 0.03 g. cod-liver oil (paste ABC), or, in some experiments, the control rats were given "normal paste" containing about 1.7 % cod-liver oil¹.

Milk was given to the rats *ad libitum*; their average consumption was as follows:

Table I.

	Beginning of experiment	End of experiment
	cc.	cc.
Fresh milk ...	37	50
Heated milk ...	35	70
Oxidised milk ...	35	58
" " + ABC paste	35	56

That is to say, on the whole, the rats consumed a slightly larger quantity of heated, and even oxidised milk than of fresh milk. The very high average

¹ The composition of N paste was the same as that used by Korenchevsky [1921, 1922].

figure of heated milk consumed may be explained by the fact that this average was obtained from three rats, the rest of the rats of this litter also consuming more than the average of fresh or oxidised milk shown in Table I (65 cc. and 63 cc. respectively). The average amount of "normal" paste consumed by the rats was about 20 g.

In the various experiments of the first series the rats were divided into the following groups (see Table II):

Table II. *Diet of rats.*

No. of experiment	FM + 3 g. BC I	HM + 3 g. BC I	OM + 3 g. BC I	OM + 3 g. ABC	"Normal" paste
36	1	—	3	1	1
37	2	—	3	2	2
38	1	—	2	—	1
39	2	—	4	—	1
40	1	1	2	—	1
41	1	2	2	1	1
58	7	—	—	—	1
Total	15	3	16	4	8

In every experiment the rats were taken from the same litter. The rats in experiment 58 were born of parents fed on FM diet, and were themselves fed on that diet after weaning.

Besides these experiments, this series includes one more experiment, No. 42, conducted on five rats, described later.

The average figures of the age of the rats, of the duration of the milk diet, as also the average chemical composition of the skeleton of the rats in experiments 36 to 41, are shown in Table III. As the results were practically identical, no tables of separate experiments are given.

Table III. *Chemical composition of the skeleton of rats fed on milk ad libitum.*

No. of experiment	Age in days		Feeding in days	FM + BC I in bones			HM + BC I in bones			OM + BC I in bones			OM + N paste in bones			OM + ABC in bones		
	Initial	Final		H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %
Exps. 40 (5 rats)	23	66	43	42.0	12.1	20.8	38.7	13.2	21.6	37.7	13.6	21.9	37.9	13.4	21.5	—	—	—
" 38 (4 rats)	17	105	88	39.7	13.2	21.9	—	—	—	37.3	13.6	21.7	37.9	14.2	22.7	—	—	—
" 41 (7 rats)	24	100	85	36.7	13.9	22.0	36.5	14.1	22.0	37.3	13.9	22.1	39.7	12.9	21.3	39.4	12.6	20.7
" 36 (6 rats)	21	118	97	37.6	13.9	22.3	—	—	—	37.6	14.1	22.7	39.4	13.0	21.5	36.7	14.5	22.5
Average of expts. 38, 41, 36	21	111	90	38.0	13.7	22.1	36.5	14.1	22.0	37.3	13.9	22.2	39.0	13.4	21.8	38.0	13.5	21.6
Exps. 37 (9 rats)	36	130	94	38.7	14.0	22.9	—	—	—	36.6	14.4	22.7	38.2	14.0	22.6	37.9	14.2	22.8
" 39 (7 rats)	40	140	100	37.7	14.1	22.6	—	—	—	38.3	13.6	22.2	40.3	12.8	21.5	—	—	—
Average of expts. 37, 39	38	135	97	38.2	14.1	22.7	—	—	—	37.5	14.0	22.5	39.2	13.4	22.1	37.9	14.2	22.8

These data show that in the different groups of rats consuming milk *ad libitum* no essential difference in the chemical composition of the skeleton was observed, even when, for instance, the oxidised milk diet was begun at a very early age, immediately after weaning (17th day after birth), and was continued for about three months.

Histological examination likewise showed no difference between the structure of the skeleton of rats fed on FM, HM or OM. Sometimes the microscopical picture of the skeleton of rats on a milk diet differed from that of rats on a normal diet only in being slightly more osteoporotic, this condition being more perceptible in rats fed on OM. The osteoporosis was due to the lowered activity of osteoblasts (the number of osteoclasts being normal).

The weight curves of rats of various categories in experiments 36-41 were identical, and in no way different from the weight curves of rats on a normal diet. In view of this, they are not shown here. There was likewise no difference in the external appearance and nutrition of the various groups of rats.

Thus the large ration of OM consumed by the rats retained sufficient of the fat-soluble factor for the growth of the rats, and the composition and structure of their skeleton were practically normal even when air was passed through the milk at a temperature of $98^{\circ}8$ C. during seven hours.

In experiment 58, the second generation of rats on FM diet showed a marked difference from the normal as regards external appearance: rats killed at the age of 57 days were some 60 % below the normal weight; in many places the fur was so thin that some rats were nearly hairless. Moreover, they all suffered from emaciation and anaemia. Chemically, the skeleton was normal in structure. Histologically, the bones showed a moderate increase of the proliferating cartilage with an insufficient deposition of calcium into the zone of provisional calcification in the case of some rats. There was considerable osteoporosis, but the amount of osteoid was not increased.

The object of experiment 42 was to ascertain the influence on the skeleton of rats of an addition to the milk diet either of vitamin B alone (in the form of a paste with yeast), or of vitamin C alone (paste with orange juice). The milk given to the rats was either fresh or oxidised.

Neither the chemical nor the histological examination of the skeleton showed any effects of the addition of vitamins B and C to the milk given to the rats *ad libitum*. The composition of the skeleton of the rats in experiment 42 was compared with that of the control rats from the same litter, fed on a "normal" diet. In view of the negative results of the experiment, we give no detailed data.

SECOND SERIES OF EXPERIMENTS. INFLUENCE OF MEDIUM DOSES OF MILK (15 TO 30 CC. PER DAY).

Thirty-nine rats, belonging to six litters, were used in this series of experiments. The milk was given to the rats in the same form as in the first series of experiments, *i.e.* FM, HM and OM; except that, for the purpose of intensifying the process of the oxidation of the fat-soluble factor, 1 cc. of a 20 % solution of hydrogen peroxide was added per 100 cc. milk. On investigation it was found that after heating the milk for seven hours and passing air through it, any reaction of hydrogen peroxide had disappeared.

The experiments were conducted from February to the first half of May, *i.e.* during the first third or half of the experimental period "winter" milk was used, and the rest of the time "spring" milk.

In this series of experiments the young rats were weaned at the age of 19 to 20 days, after which, for a period of from 13 to 34 days (Period I), they were fed exclusively on OM. From the age of 33–35 days the rats were transferred to a diet consisting of a moderate ration of milk (15 to 30 cc.) (Period II). The number of calories taken in by each rat was equalised by the starch given to the rats in pastes BC II and BC III. In these pastes the rats received approximately the same amount of vitamins B and C in the daily dose. The composition of the paste was as follows:

			Paste BC II	Paste BC III
Starch	61.5 g.	66 g.
Yeast	3.5 g.	5 g.
Orange juice	5.0 cc.	6 cc.
Water	30.0 cc.	23 cc.

To the daily ration of 15 cc. milk was added 16 g. of paste BC II; to 30 cc. milk 11 g. of paste BC III.

For purposes of comparison some rats received, even during Period II, large doses of milk with 3 g. of paste BC I, or 3 g. of paste ABC (see p. 190).

During Period II the rats were fed for 86 to 103 days, after which they were killed at ages varying from 122 to 151 days.

Thus, during Period II, the rats in the experiments of the second series, as compared with those of the first series, received a considerably smaller amount of protein, fat, salts, and fat-soluble factor.

In the various experiments of the second series the rats were divided into the following groups (see Table IV):

Table IV. *Diet of rats during Period II.*

No. of exp.	50 cc. FM + 3 g. BC I	15 cc. FM + 16 g. BC II	50 cc. OM + 3 g. BC II	50 cc. OM + 3 g. ABC	15 cc. OM + 16 g. BC II	30 cc. OM + 11 g. BC III	30 cc. OM + 11 g. BC III + 0.03 g. cod- liver oil
52	—	2	—	1	4	—	—
53	1	2	—	1	3	—	—
54	—	—	—	—	2	2	2
56	1	2	1	—	2	—	—
57	1	2	1	—	2	—	—
Total	3	8	2	2	13	2	2

Besides this, in experiment 55 there were seven rats the distribution of which will be mentioned later, in describing that experiment.

In experiments 52, 53, 54, 56 and 57, during Period I, *i.e.* that of an exclusive diet of OM, the first few weeks after weaning the rats consumed, on an average, about 23 cc. of milk per diem, and later about 40 cc. Those rats which received milk *ad libitum* during the whole experimental period, consumed about 50 cc. during the last third of the time, there being no difference in the consumption of FM and OM. The ration of 15 to 30 cc. of

milk and pastes BC II and BC III was completely consumed by the rats, with the exception of one rat, fed on OM.

There was very little difference in the appearance of the rats of each group. The curves of the average weight of the rats of each group in these experiments are shown in Fig. 1 (males) and Fig. 2 (females). The weight curves of rats on OM are somewhat below those of rats on FM, this being more marked in the case of rats on a ration of 15 cc. milk. A similar, merely slight, retardation of the weight curves was observed in the case of rats fed on 15 cc. milk, as compared with those of rats receiving and consuming about 50 cc. milk. The age, duration of feeding on special diets, and the chemical composition of the skeleton of rats in the experiments described are given in Table V. This table shows the following:

Table V. *Chemical composition of the skeleton of rats fed on 15-50 cc. of milk.*

No. of exp.	Age in days			Duration of feeding in days			50 cc. FM+ABC in bones			50 cc. FM+BC I in bones			50 cc. OM+ABC in bones			50 cc. OM+BC I in bones			15 cc. FM+BC II in bones			15 cc. OM+BC I in bones		
	When put on OM only	When put on milk and paste	Final	On OM only	On milk and paste		H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %	H ₂ O %	Fresh Ca %	Dry Ca %
Exp. 52 (7 rats)	19	53	146	34	93	—	—	—	—	—	—	—	35.2	14.7	22.6	—	—	—	35.1	13.7	21.1	30.0	13.3	20.0
Exp. 53 (7 rats)	19	54	151	35	97	31.4	16.3	23.7	—	—	—	—	32.5	14.6	21.7	—	—	—	32.7	14.7	21.9	35.3	14.5	21.0
Exp. 54 (2 rats)	20	45	144	25	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30.9	12.1	20.0
Average of exps. 52 and 53	19	54	149	35	95	31.4	16.3	23.7	—	—	—	—	33.8	14.7	22.2	—	—	—	33.9	14.2	21.5	37.1	13.3	20.0
Exp. 56 (6 rats)	20	33	133	13	100	—	—	—	32.6	15.9	23.7	—	—	—	—	32.1	14.9	22.0	35.4	13.7	21.2	34.4	14.0	21.0
Exp. 57 (6 rats)	20	33	136	13	103	—	—	—	30.5	15.5	22.4	—	—	—	—	34.1	13.8	20.9	34.1	14.1	21.3	38.9	12.7	20.0
Average of exps. 56 and 57	20	33	135	13	102	—	—	—	31.6	15.7	23.0	—	—	—	—	33.1	14.3	21.4	34.7	13.9	21.3	36.6	13.4	21.0

On an OM diet, a slight diminution of calcium in the skeleton was observed: *e.g.* on 50 cc. OM the skeleton, as compared with that of rats on FM, was more deficient in dry bone (about 7 %) and in fresh bone (9 %); on a diet of 15 cc. milk it was not in every experiment that even this slight difference was observed. In any case, the difference seen in the composition of the skeleton was so slight that it might have been due to physiological fluctuations. Histologically, there was also no considerable difference between the structure of the skeleton of rats in different groups. Only a slight degree of osteoporosis might be observed in the rats especially fed on OM.

Experiment 55 was conducted in such a manner as to approximate the conditions to those of bringing up children by hand, that is to say, the rats were given diluted milk *ad libitum*. The requisite number of calories was provided by a 17 % solution of cane sugar, with which the milk was diluted. The milk was heated in a bath of boiling water for seven hours. The seven

rats used in this experiment were divided into four groups, and at the age of 36 days were placed on the following diets:

Table VI.

Denomination of milk	No. of rats	Dilution of milk: Percentage of 17 % solution of cane sugar contained in milk	Consumption of milk in cc.	
			Beginning of experiment	End of experiment
HM	2	—	27	46
DM ₁	2	60	22	50
DM ₂	2	40	23	50
DM ₃	1	50	24	28

Besides milk, each rat received 3 g. of paste BC 1. After 86 days of feeding on the above-mentioned diets, the rats were killed. The rats fed on DM₁ and DM₂ were slightly (about 8 %) lighter in weight than those on HM, but, on the whole, were not different from them in any other respect. The rat on DM₃ weighed 37 % less than the rats on HM, and was thin. As may be seen from Table VI, its appetite was worse than that of any of the others.

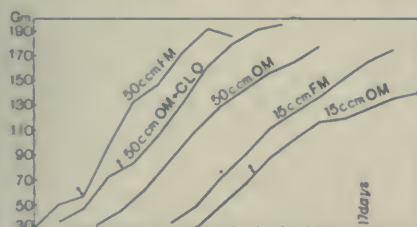


Fig. 1. Weight curves of average rats (males) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

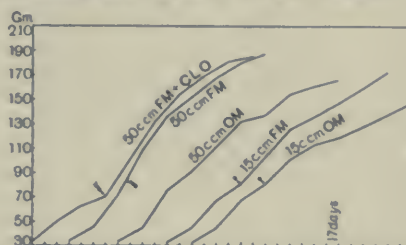


Fig. 2. Weight curves of average rats (females) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

In all the rats fed on diluted milk, as compared with the rats fed on HM, the composition of the skeleton was worse: the average water content of the bones was 8 % greater, the calcium content in fresh bone was about 15 % less, and about 12 % less in dry bone. Histologically, the rats fed on diluted milk, as compared with those fed on HM, showed more marked osteoporosis, and in some cases there was a slight enlargement of cartilage and an irregular line of osseo-chondral junctions. A slightly increased amount of osteoid was observed only in the case of one rat fed on DM₃.

Thus, in this series of experiments also, no considerable skeletal changes could be obtained. It is a striking fact that the seven-hours' aeration of milk at 98.8 C. did not diminish the amount of the fat-soluble factor in the milk to such an extent that the growth of the rats and the calcification of their skeleton was affected considerably, even by the ingestion of only 15 cc. of milk per diem. On the other hand, larger quantities of milk (up to 30 cc.), diluted with 17 % sugar solution, caused greater skeletal changes, that is to say, apparently an exclusively liquid diet has an unfavourable effect on animals if continued for a long time.

THIRD SERIES OF EXPERIMENTS. INFLUENCE OF SMALL DOSES OF MILK (5 CC.) ON THE SKELETON OF RATS FED ON A DIET DEFICIENT IN THE FAT-SOLUBLE FACTOR.

In this series there were 40 rats, belonging to five litters. In each experiment the rats, after being put on a diet deficient in the fat-soluble factor (—A diet)¹ for 33–39 days, were divided into the following groups:

- Group I remained on —A diet;
 „ II transferred to normal diet (N)¹, containing butter and cod-liver oil as a source of fat-soluble factor;
 „ III fed on —A diet + 5 cc. FM;
 „ IV fed on —A diet + 5 cc. HM; and
 „ V fed on —A diet + 5 cc. OM.

The distribution of the rats into various groups in each experiment, their weight, age, duration of various dieting, the chemical composition of their skeleton and the histological diagnosis, are shown in Tables VII–XI.

The amount of N paste consumed by the rats fluctuated between 16 and 21 g.; the amount of —A paste consumed per diem varied in the different experiments: in experiments 43 and 44 from 10 to 14 g. were eaten; in experiment 45 from 9 to 11 g., but in the case of rats fed on FM and of one (490) fed on HM, the paste was consumed in larger quantities—14 to 15 g.; in experiment 47 the average consumption reached 10 g.; in rats fed on FM it was 11 g. Thus with some rats the ingestion of fresh milk alone increased their appetite.

Table VII.

Experiment 43. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on —A diet without milk 38 days (period I), with milk 20 days (period II). Final age: 96 days.

No. of rat	Sex	Diet in period II	Weight in g.			In bones			Histological results
			When put on —A diet	At the beginning of period II	Final	H ₂ O %	Fresh Ca %	Dry Ca %	
474	♂	N + FM	43	58	132	38.9	13.0	21.2	*Normal
477	♀	—A + FM	51	70	97	47.8	8.7	16.6	Osteoporosis
480	♂	—A + FM	46	74	95	50.0	7.5	15.1	Slight rickets and osteoporosis
Average of 477 and 480:—						48.9	8.1	15.9	
475	♀	—A + HM	56	73	98	46.2	8.4	15.5	Slight osteomalacia
476	♂	—A + OM	55	75	92	48.0	7.7	14.7	Slight osteomalacia
479	♂	—A + OM	48	64	87	47.5	7.3	14.4	Slight rickets
Average of 476 and 479:—						47.7	7.5	14.6	
478	♂	—A alone	53	84	87	51.3	7.5	15.4	Slight rickets. Osteoporosis. Died from cachexia 3 days before the end of the experiment

* Analyses lost; these figures of the chemical composition are taken from rat 501 which was of a corresponding age. (See also very similar figures from rats aged 80–120 days [Korenchevsky, 1922, 3, p. 65].)

¹ For the composition of the diets N and —A, see previous communications [Korenchevsky, 1921, 1922].

Table VIII.

Experiment 48. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on - A diet without milk 33 days (period I), with milk 26 days (period II). Final age: 104 days.

No. of rat	Sex	Diet in period II	Weight in g.			In bones			Histological results
			When put on - A diet	At the beginning of period II	Final	H ₂ O %	Fresh Ca %	Dry Ca %	
510	♀	- A + FM	62	72	110	43.5	9.8	17.3	Slight rickets
513	♂	- A + FM	62	78	132	49.4	8.3	16.5	Slight rickets, osteoporosis
Average of 510 and 513:—						46.5	9.1	16.9	
507	♀	- A + HM	69	60	89	48.1	8.5	16.5	Slight rickets, osteoporosis
511	♀	- A + HM	85	96	128	42.9	10.4	18.3	Slight rickets
Average of 507 and 511:—						45.5	9.5	17.4	
508	♀	- A + OM	63	72	87	48.1	8.1	15.6	Slight rickets, osteoporosis
512	♂	- A + OM	88	120	144	45.5	9.5	17.4	Slight rickets
Average of 508 and 512:—						46.8	8.8	16.5	
509	♂	- A alone	85	112	128	45.7	8.4	15.5	Moderate rickets

Table IX.

Experiment 47. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on - A diet without milk 39 days (period I) with milk 48 days (period II). Final age: 109 days.

501	♀	N + FM	47	59	158	38.9	13.0	21.2	Normal
502	♂	- A + FM	49	70	113	51.0	8.6	17.5	Very slight rickets
506	♂	- A + FM	56	74	137	49.2	8.5	16.7	Slight rickets
Average of 502 and 506:—						50.1	8.6	17.1	
504	♂	- A + HM	57	70	118	50.4	6.9	13.8	Slight rickets, osteoporosis
500	♂	- A + OM	54	70	117	53.3	6.6	14.0	Moderate rickets
503	♀	- A + OM	41	63	65	56.7	6.7	15.6	Slight rickets. Osteoporosis.
Average of 500 and 503:—						55.0	6.7	14.8	Hyperaemia of bone marrow. Died from cachexia
505	♀	- A alone	43	62	81	49.5	7.6	15.0	Moderate rickets

Table X.

Experiment 45. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on - A diet without milk 35 days (period I), with milk 63 days (period II). Final age: 139 days.

493	♀	N + FM	35	55	180	38.1	13.3	21.5	Normal
494	♂	N + FM	36	60	248	44.3	11.7	21.0	Normal
Average of 493 and 494:—						41.2	12.5	21.2	
485	♀	- A + FM	43	79	106	41.2	11.2	19.0	Slight rickets
488	♂	- A + FM	32	68	163	45.2	8.9	16.2	Slight rickets
Average of 485 and 488:—						43.2	10.0	17.6	
486	♀	- A + HM	34	58	103	49.0	7.8	15.3	Slight rickets
490	♂	- A + HM	34	75	162	46.7	8.3	15.7	Slight rickets
Average of 486 and 490:—						47.8	8.1	15.5	
484	♀	- A + OM	35	65	94	44.5	8.0	14.3	Slight rickets
491	♂	- A + OM	33	74	125	44.0	7.3	13.1	Slight rickets, osteoporosis
492	♀	- A + OM	37	62	99	50.4	7.5	15.0	Moderate rickets
Average of 484, 491 and 492:—						46.3	7.6	14.2	
487	♂	- A alone	35	71	82	50.9	6.4	13.1	Severe rickets
495	♂	- A alone	47	86	117	47.6	7.3	14.0	Moderate rickets, osteoporosis
496	♀	- A alone	56	84	122	49.9	6.9	13.9	Severe rickets
Average of 487, 495 and 496:—						49.5	6.9	13.6	

Table XI.

Experiment 44. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on — A diet without milk 38 days (period I), with milk 66 days (period II). Final age: 144 days.

No. of rat	Sex	Diet in period II	Weight in g.			In bones			Histological results
			When put on — A diet	At the beginning of period II	Final	H ₂ O %	Fresh Ca %	Dry Ca %	
445	♀	N + FM	57	67	143	40.3	12.2	20.5	Normal
451	♀	— A + FM	49	62	105	43.5	11.2	19.8	Nearly normal
447	♀	— A + HM	67	104	134	44.6	10.1	18.3	Slight rickets
449	♀	— A + HM	58	71	103	41.4	11.0	18.7	Nearly normal
Average of 447 and 449:—						43.0	10.5	18.5	
448	♂	— A + OM	60	78	151	40.8	10.3	17.6	Slight rickets
450	♂	— A + OM	54	84	133	49.5	8.9	17.7	Slight rickets
Average of 448 and 450:—						45.2	9.6	17.7	
446	♂	— A alone	66	87	99	47.2	8.8	16.6	Slight rickets. Moderate osteoporosis

The weight curves of rats are given in Figs. 3–6, and show that the addition of 5 cc. milk to — A diet improved the growth of the rats. Moreover, the greatest effect was produced by FM, to a lesser degree by HM, the least effect being produced by OM; that is to say, the mere heating of the milk for seven hours at 100° C. in most cases caused a slight disintegration of the growth-promoting properties of milk, but the greatest effect was produced by oxidation combined with heating. At autopsy, the following peculiarities were observed in the various groups of rats: on N diet the rats were fat and normal; on — A diet they were thin or emaciated, with opaque teeth, frequently with depressed thorax and numerous fractures of the ribs. In some rats the ribs and the bones of their hind legs were curved.

On — A diet + 5 cc. OM the macroscopical changes hardly differed from those on — A diet alone, the only difference being that the rats were somewhat better nourished.

Table XII. *Summary table showing in percentage the influence of small doses (5 cc.) of milk on the chemical composition of the skeleton of rats kept on — A diet.*

No. of exp.	Above (+) or below (–) — A standard. In bones											
	N + FM			— A + FM			— A + HM			— A + OM		
	H ₂ O	Fresh Ca	Dry Ca	H ₂ O	Fresh Ca	Dry Ca	H ₂ O	Fresh Ca	Dry Ca	H ₂ O	Fresh Ca	Dry Ca
I. Milk given during a period of 20–26 days (average 23 days).												
Exp. 43 (7 rats)	–24.2	+73.4	+37.5	–4.7	+8.0	+2.7	–10.1	+12.0	+0.8	–7.0	–4.0	–5.6
„ 48 (7 rats)	—	—	—	+1.6	+7.6	+9.0	–0.5	+12.8	+12.1	+23.6	+4.4	+6.5
Average of exps. 43 and 48	–24.2	+73.4	+37.5	–1.6	+7.8	+5.9	–5.3	+12.4	+6.5	+8.3	+0.2	+0.45
II. Milk given during a period of 49–66 days (average 60 days).												
Exp. 47 (7 rats)	–21.3	+70.9	+41.2	+1.3	+12.4	+13.7	+1.9	–9.5	–7.8	+11.2	–12.3	–1.2
„ 45 (12 rats)	–16.7	+81.3	+55.6	–12.6	+45.4	+28.9	–3.3	+17.1	+13.9	–6.4	+10.0	+3.4
„ 44 (7 rats)	–14.6	+39.0	+23.4	–7.8	+27.3	+19.6	–8.9	+19.8	+11.5	–4.4	+9.6	+6.5
Average of exps. 47, 45 and 44	–17.5	+63.7	+40.1	–6.3	+28.3	+20.7	–3.4	+9.1	+5.9	+0.1	+2.4	+2.9

On $-A$ diet + 5 cc. FM the macroscopical picture showed a marked difference as compared with $-A$ diet: the rats were considerably better nourished, the skeleton was either normal or, if there were calluses after spontaneous fractures, they were solitary cases, and were far smaller than those in rats on $-A$ diet alone or with OM. On $-A$ diet + 5 cc. HM the macroscopical changes in rats were of a medium character between those of rats fed on OM and FM, being more like the latter. The chemical changes shown in Tables VII-XI are summarised in Table XII. It will be seen from

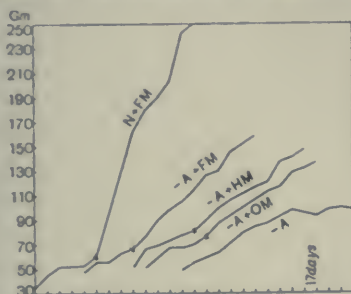


Fig. 3. Weight curves of average rats (males) from experiments 44, 45 and 47. Up to the point marked \times rats were kept on $-A$ alone. The diet after that is shown above each curve.

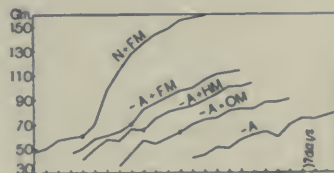


Fig. 4. Weight curves of average rats (females) from experiments 44, 45 and 47. Up to the point marked \times rats were kept on $-A$ alone. The diet after that is shown above each curve.

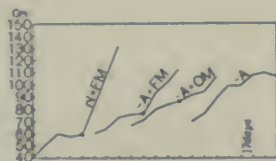


Fig. 5. Weight curves of average rats (males) from experiments 43 and 48. Up to the point marked \times rats were kept on $-A$ alone. The diet after that is shown above each curve.

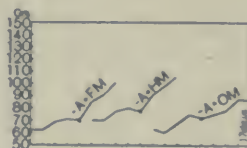


Fig. 6. Weight curves of average rats (females) from experiments 43 and 48. Up to the point marked \times rats were kept on $-A$ alone. The diet after that is shown above each curve.

this table that after milk was given for 20-26 days, these changes were only slight, being far more marked after a period of about two months. That is to say, in order for milk to produce a favourable effect, it is necessary that it should be ingested for a considerable period of time. On the whole, the most marked improvement in the chemical composition of the skeleton was observed on giving FM, followed by HM. The addition of OM to $-A$ diet either produced no improvement whatever in the chemical composition of the skeleton or had only a very slight effect. In any case, the addition of 5 cc. milk could not have caused such an improvement in the skeleton in the course of 66 days, as was produced by butter *plus* cod-liver oil in N diet.

On histological examination, the most marked improvement in rickets was observed in the case of a diet of FM or HM. On OM this improvement was usually (though not always) observed, but frequently it was less marked than in the case of FM.

Thus in this series of experiments likewise, the macroscopic, microscopic, and chemical examinations of the skeleton coincided with the data obtained in investigating the growth and weight of animals.

SUMMARY.

In our experiments the rats grew and developed normally, and had a normal skeleton, on a diet of milk *ad libitum* with the addition of the requisite amount of calories in the form of carbohydrates. They were, however, not very fertile and, if they did bear young, the latter became anaemic and cachectic on a milk diet. Though the calcium content of the skeleton of the young was *normal*, *histologically* there was *osteoporosis*, and even one of the features of rickets (enlargement of the cartilage).

The dilution of heated milk with a 17 % solution of sugar resulted in the composition of the skeleton being worse than that of rats fed on smaller quantities of milk, but with the addition of calories in the form of starch paste. The addition of even a small quantity of milk (5 cc.) to —A diet produced an improvement in rickets caused in rats by a diet deficient in the fat-soluble factor. Such a favourable action of milk was slightly decreased by heating the milk for seven hours at 100° C., and was decreased in a marked degree after the aeration of the milk at 98°·8 C. for the same period of time.

Thus these experiments show that oxidation of milk also reduces its anti-rachitic and growth-promoting properties, as was observed by Hopkins [1920] and Mellanby [1921] in the case of butter. The experiments with larger doses of milk (15 to 50 cc. per diem) have however shown that the oxidation of milk produces *only a partial*, and not total disintegration of the fat-soluble factor, and that in such larger doses oxidised milk produced nearly the same or identical growth of the rats and calcification of the skeleton as was caused by fresh milk. The addition of cod-liver oil to large doses of oxidised milk in most cases had no effect on the results, or if it did affect them, they were only slightly improved.

Thus, in our experiments we failed to obtain such a great disintegration of the fat-soluble factor as to produce rickets in rats fed on an abundant milk diet. No doubt if the oxidation had continued for a longer period, or had taken place at a higher temperature, the results would have been different.

In conclusion, it is necessary to note certain peculiarities obtained on oxidising the fat-soluble factor in milk, as compared with the results of oxidising this factor in butter or cod-liver oil.

Hopkins [1920], Drummond and Coward [1920], and Zilva [1920] showed that the growth-promoting properties of butter are easily disintegrated by oxidation. Mellanby found that oxidation likewise disintegrates the anti-rachitic properties of butter, but not of cod-liver oil. McCollum, Simmonds, Becker and Shipley [1922] found that on oxidising cod-liver oil for 12–20 hours at the temperature of boiling water, its anti-rachitic properties may be preserved,

while its capacity of curing xerophthalmia is lost. On these grounds the American authors have come to the conclusion that the so-called "fat-soluble vitamin A" contains two separate vitamins: (1) an "anti-rachitic vitamin," which is disintegrated with difficulty by oxidation, and (2) "vitamin A" itself, far more easily disintegrated by oxidation, and preserving animals from xerophthalmia.

In our experiments with oxidised milk, the following facts should be noted:

(1) Apparently the fat soluble factor is disintegrated by oxidation with greater difficulty in milk than has been observed in the case of butter. This peculiarity may be to a certain extent explained by the fact that in making butter from milk only part of the growth-promoting factor, present in milk, is transferred to the butter. Thus, according to Drummond, Coward and Watson [1921, p. 544]: "Fresh milk from cows fed on green pasture will, in nearly all cases, induce a resumption of growth in rats fed on a diet deficient in vitamin A, when given in a daily supplement of 2 cc. [cf. Hopkins, 1912, 1920]. This amount of milk may be regarded as supplying approximately 0.1 g. of fat per day. In order to induce the same amount of growth by adding a supplement of butter, it is necessary to give about 0.2-0.3 g. per day."

Certain hypotheses may be advanced with regard to this. For instance, McCollum [1917] thinks that water solutions of the non-lipoid constituents of milk contain this substance (vitamin A), indeed, in considerable amount. His experiments lead him to believe that approximately half of the fat-soluble dietary essential is present in the fat and half in the non-fat portion of the milk. Another hypothesis supposes a partial loss of the fat-soluble factor to occur during the churning of the butter (perhaps due to oxidation). If in milk there is a larger amount of fat-soluble factor than in butter, we assume, that to destroy this factor in milk will take longer and be more difficult.

(2) In our not numerous experiments the mere heating of milk at 100° C. for seven hours almost invariably slightly decreased the growth-promoting and anti-rachitic properties of milk (a phenomenon observable on adding 5 cc. HM to -A diet). This cannot be explained by the separation of lime salts in heating the milk¹. An investigation of the Ca content in OM did not show any essential alteration in it. Therefore our experiments do not support the hypotheses of Daniels and Loughlin of the decrease in the calcium content of milk as a result of boiling or heating the latter (provided the milk is given to the rats after having been shaken). Finally it may be possible that by heating the milk the calcium compounds are altered to those less able to be assimilated by the organism. However, such a small amount of calcium as is found in 5 cc. of milk and added to -A diet could not influence the composition of the skeleton.

It is known, that merely heating butter or cod-liver oil has no effect on the content of fat-soluble factor in either [Osborne and Mendel, 1915; Hopkins, 1920; Mellanby, 1921]. Therefore, if subsequent and more numerous experi-

¹ The milk was shaken thoroughly before being given to the rats or taken for analyses.

ments confirm the fact noticed by us, it may be found that milk contains, besides a fat-soluble factor, another special factor which to some extent is disintegrated by heating.

(3) In our experiments we did not observe any considerable decrease in the growth-promoting properties of milk, without simultaneous loss of its anti-rachitic properties. Almost invariably there was a parallel decrease in the above-mentioned properties after oxidation. This fact does not agree with McCollum's observations on the relative decrease after oxidation of anti-xerophthalmic and anti-rachitic vitamins in cod-liver oil, though it is in accordance with Mellanby's experiment with butter referred to above.

At present it is difficult to explain this peculiarity, and it is only possible to form conjectures:

(a) Taking the point of view of McCollum and co-workers, we may assume that butter contains a small amount of the anti-rachitic factor, while its vitamin A content is large. The great degree of oxidation required to disintegrate the large amount of vitamin A may at the same time disintegrate a small quantity of the anti-rachitic vitamin, *e.g.* in butter. Possibly, the enormous amount of anti-rachitic vitamin contained in cod-liver oil has no time to be inactivated by the degree of oxidation which is sufficient to destroy the anti-xerophthalmic and growth-promoting properties.

(b) It is possible that the presence of milk fat in whey, in the form of an emulsion, alters the conditions of the oxidation of the fat-soluble factor.

(c) In the same way, this fact may, to a certain extent, be explained by the existence of a special factor in milk, different from the fat-soluble factor.

CONCLUSIONS.

(1) When rats are fed on fresh, heated or oxidised milk *ad libitum*, supplemented by carbohydrates, the structure of their skeleton hardly differs from the normal, either chemically or histologically. When oxidised milk is used only a slight degree of osteoporosis in the bones is observed.

(2) Fresh winter milk, as supplied to us by a London Dairy, even to the amount of 5 cc., causes a considerable improvement in rickets induced in rats by -A diet, and a renewal of growth that has been inhibited.

(3) Heating the milk for seven hours at 98.8-100° C., with or without aeration, produces no essential change in its calcium content.

(4) When milk is oxidised at 98°-8 C. for seven hours, its growth-promoting and anti-rachitic properties are obviously decreased, as is seen from experiments with small doses (5 cc.) of milk.

(5) Nevertheless, these properties are so far retained that, on the daily ingestion of 15 cc. and more of oxidised milk by rats for a period up to 132 days, the rats hardly differ, in growth and skeletal development, from those fed on fresh milk.

(6) Apparently, the deficiency of the cow's food in the fat-soluble factor induces a far greater impoverishment of the milk in that factor than the addition of 1 % of peroxide of hydrogen to the milk, and subsequent aeration for seven hours at 98°-8 C.

(7) Heating the milk for seven hours at 100° C. slightly decreases the growth-promoting and anti-rachitic properties of the milk.

A grant from the Medical Research Council and the hospitality of the laboratories of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

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XXV. ON THE EFFECT OF DEFICIENCY OF IRON IN THE DIET OF PIGS.

(PRELIMINARY COMMUNICATION.)

By JOHN POOL MCGOWAN AND ARTHUR CRICHTON.

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(Received February 16th, 1923.)

IN a previous paper [Elliot, Crichton and Orr, 1922], the importance of the inorganic constituents of food in nutritional disorders, with especial reference to rickets in pigs, was pointed out. In this paper, attention will be drawn to the important place that another inorganic constituent, namely iron, may take in the growth and development of the same animals.

In a large breeding establishment for pigs, it was the custom to keep the sows out in the open, on pasture, till within a fortnight of their farrowing date. They were then brought into the house and put in pens with cement floors, walls etc. Their food, when in the house, consisted of fishmeal, bruised maize, and "draff" or brewers' offal in certain proportions, together with an abundant supply of water.

This diet contained very little iron. The fishmeal was of the white variety, prepared from the bones and adherent flesh of white fish, without the blood, intestines or other organs and contained only a trace of iron. (Albu-Neuberg [1906] found only a trace of iron in anchovies.) Maize contains 0.008 % and barley, from which "draff" is obtained, 0.024 % iron [Forbes, 1909, p. 152].

The sows farrowed in due course and the pigs appeared to do well till they were from three to four weeks old. Then the following train of symptoms began to appear. The pigs became dull and listless, their skins became intensely white, they developed a hairy appearance, and they took on a "stocky" or thick-set look (due to oedema of the skin). In addition to these, the breathing became pumping in character and "thumps" or spasmodic jerking of the diaphragm developed. Sudden death became a common event, until whole litters perished one after the other. Cases would be seen standing up alive and, in a few minutes, they would be seen in respiratory convulsions on the floor.

On *post mortem* examination of cases at this stage, the following appearances would be found. The animals were in an apparently very fat condition, subcutaneous fat being specially abundant. The heart would be found so dilated as actually to fill almost the entire chest cavity, the lungs being crushed back and collapsed until they were confined in the angle between the bodies of the vertebrae and the ribs. There was great effusion into the pericardial cavity, as also into the pleural cavity, and the lungs were oedematous. There was great effusion of fluid also into the peritoneal cavity, and

this fluid contained large coagulated flakes. The liver was pale in colour and showed, all over its surface and substance, minute paler areas of the size of a millet seed. These areas were in the centre of the lobules. The pancreas was extremely white in colour, resembling a piece of chalk. The kidneys were also pale, and the spleen in most cases was slightly enlarged.

The blood and tissues were sterile. The blood was extremely watery and pale, the haemoglobin in many cases being about 15 %, and the red blood corpuscles about 3,000,000 per mm³.

Microscopic examination of the tissues showed extreme fatty change in the centre of the lobules of the liver. There was also advanced fatty degeneration of the heart muscle and of the epithelium of the secreting tubules of the kidneys. (The urine contained a very slight trace of albumin.)

In the cases which continued to live, and these were few if the disease was at all advanced and if treatment was not adopted, the following appearances developed. The pig became emaciated and ceased to grow; it became very hairy and the skin assumed a cinnamon tint and became very dry and dirty. The animal became very apathetic and no longer desired food. Many of them died subsequently, and on *post mortem* examination of such cases the following appearances were found. Owing to the resorption of the effused fluid in various parts of the body, there were adhesions between the heart and the pericardium, the pericardium and the lungs and the chest wall. The lungs were collapsed and bound down with adhesions to the angle between the bodies of the vertebrae and the ribs. Often there was a pneumonic condition in their dependent margins. The lungs were also adherent to the upper surface of the diaphragm. In the abdomen the diaphragm was fixed by adhesions to the upper surface of the liver, the various lobes of the liver being also adherent to one another and to the coils of the intestine. These latter also were attached to the spleen and to one another. Especially was this seen in the large intestine, where the coils were gummed together leaving practically no furrow between them.

The condition of matters, just described, added to the mortality caused by an epidemic of distemper in the herd on account of the lung complications developed. It, alone or in conjunction with the distemper, gave rise to an outbreak of abscesses in the face, due to caries of the temporary teeth.

The following would appear to be the explanation of the train of events just described. While the sows were out at grass, they obtained abundant iron from the grass and the soil. When, however, they were taken into the pens a fortnight before farrowing, owing to the cement floors and the nature of the food, the supply of iron ceased. In spite of this, presumably owing to a certain amount of iron stored up, they were able to carry on for a period, but, after a time, this supply was insufficient for themselves and the requirements of the growing litters of pigs. The result was that the symptoms and effects of iron deficiency as described appeared in the pigs, and the sows themselves became very emaciated. In this latter connection, it may be

mentioned that the pigs of those sows which contrived in some way to keep up their condition suffered relatively less from the disease.

The milk of swine contains 0.009 % of iron as compared with 0.002 % in the cow, thus showing a high demand in the pig [König, 1920]. There is a storing up of iron in the body of the unborn mammal in anticipation of the poverty of milk in this essential nutrient. During the whole of the suckling period, the young animal is drawing upon this reserve and lowering the percentage in its body [Bunge, 1889]. Hess, Unger, and Supplee [1921], show that the iron content of milk varies with the feed of the animal. Thus cow's milk contains twice as much iron when the animals are fed on pasture as when they are fed on a mixture of bean meal, linseed meal, hominy, gluten meal, and bran together with dried beet pulp, molasses and straw (an anti-scorbutic-free ration).

At the time when it became evident that deficiency of iron was the cause of the disease, there were in the piggery two groups of pigs, first, those over three weeks old, already in the grip of the disease with the organic changes in the liver, heart, kidneys and lungs, and second, those under three weeks old down to those newly born, in which the disease had not begun or where at least it was not so advanced. The mothers of both sets, as well as those pigs which were already feeding for themselves, were at once put on to large doses of ferric oxide, which was administered in their food. The effect of this was noticeable almost at once in several directions. The sudden deaths ceased at once. The pigs became more lively and had more appetite for their food, those of them, which had arrived at the stage of feeding for themselves, now squealing whenever they heard a bucket rattled. Examination of the blood of about a hundred of them, before and after the administration of the iron, showed that the haemoglobin had risen in about three weeks from 20-30 % to 70-80 %. This return of the haemoglobin to about normal did not, and could not be expected to, remove the disease symptoms entirely, owing to the organic changes in the liver, lungs and elsewhere which its lack had produced. No amelioration could for instance be expected in the case of the collapsed adherent lungs, the adherent pericardium and the adhesion of the various organs in the abdomen. The iron, however, had a marked effect in removing the fatty changes in the various organs. This was especially noticeable in the liver, which became normal in consistence and colour, except where, in some cases, the process of regeneration of the liver tissue could be seen in raised darker coloured ring-like areas—like "fairy rings"—on the surface and in the substance of the liver.

The effect of the administration of the iron to sows, before their pigs became visibly affected with the disease, may be judged of by the fact that, apart from the cessation of the sudden death condition, the pigs now were normal and healthy and the pigs of such litters at eight weeks old were about three times the size of the diseased pigs at fourteen weeks old (37 pounds as compared with 14 pounds).

The disease described here would appear to be the same as that discussed

by (amongst others) Wither and Carruth [1918] as cotton seed poisoning. The symptoms and *post mortem* appearances are alike and Wither and Carruth used iron salts to treat the disease with beneficial results. They did not however use iron salts with the idea that the condition was due to a deficiency of iron but apparently with the view that "the iron salts combine with or facilitate the oxidation of the harmful substances in the cotton-seed meal." The only other reference in the literature which we can find bearing on what may be this disease, occurs in Hutyra and Marek [1913] where a description is given of a disease called enzootic hepatitis of pigs. The disease apparently occurs when the pigs are from two to four months old and the pathological appearances, so far as they are mentioned, resemble closely those of the disease we have described. It caused great losses in Russia and during the year 1906 "the losses from this disease in Eastern Prussia were greater than those due to Swine Plague."

The disease discussed in this paper is a very important one in the pig industry. In the instance we have described it occurred not as an experimental production, but in the ordinary course of events in a pig rearing undertaking and we have found evidence of its occurrence in a considerable number of other like concerns. Its appearance depends on the nursing sows being fed on food which for various reasons does not contain enough iron to keep pace with the demands of the growing pigs. The subject matter of this investigation may have an important bearing on the pathology of chlorosis and on the pathology and aetiology of "Wet" Beri-beri in human beings. The fact that in the latter condition the symptoms and *post mortem* appearances are very similar to those described above in the pigs and that while rice bran contains 0.232 % of iron, polished rice contains only 0.003 % [Forbes, 1909, p. 152] is suggestive in this connection. The problem is being further inquired into from these directions, as also from the general stand-point and its relation to the cotton-seed poisoning so prevalent in the United States.

Orr [1922] has emphasised the importance of the mineral content of the food in connection with the etiology of rickets. He has not however specifically mentioned iron. Sollmann [1907] points out that human milk contains about three times as much iron as the artificial foods commonly fed to infants. It is probable that deficiency of iron may be a factor in conditions of malnutrition in infants as well as calcium and phosphorus.

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XXVI. THE TECHNIQUE OF BREEDING RATS FOR FEEDING EXPERIMENTS.

By GLADYS ANNIE HARTWELL, ELSIE CHARLOTTE MOTTRAM
AND VERNON HENRY MOTTRAM.

(Received February 19th, 1923.)

Introduction. The importance of good stock for experimental purposes cannot be exaggerated. That control material should be identical with that used for experiment is axiomatic. The elimination of variations due to disease or nutrition is of such value to the experimenter on growth problems—so fundamental to his work—that we venture to publish the means we have found the best for producing good material. The work was begun by one of us (E. C. M.) in the Biochemical Laboratory in Cambridge; carried on by another (V. H. M.) in the Animal Nutrition Department at Lever Bros. and the experience gained¹, grafted on that of the third (G. A. H.) and brought to its present development by her at King's College for Women (Social and Household Science Department). Our observations and experiments have extended over more than six years.

That the present technique produces large animals is seen from the curves of growth of the animals compared with Donaldson's curves (Figs. 1 and 2). This size is not due to fat alone for it starts from birth. Nor does it interfere with reproduction, for the animals are prolific and have large sized litters. Only in the winter months does the number per litter fall below six and that rarely. In the height of the two breeding seasons it is often over twelve. Yet the mother rat is able to bring up all the litter with a high average weight (*e.g.* with 9 in litter the average weight at the 21st day is 27.6 g.; 11 in litter, 28 g.; 12 and 14 in litter, 24 and 25 g. Donaldson gives 21 g.). The animals are almost free from intercurrent disease—a fact readily acknowledged by those who take our surplus animals. Most important is that the growth curves for any one time in the year are nearly constant. The animals show the marked vivacity, the prompt reaction to auditory stimulus and the glossy thick coats of healthy rats.

Stock. The original plan² adopted was to buy mixed stock from the dealers, keep it under observation for some time and then to breed from the does, using large and powerful bucks. This we now consider unwise. The mixed

¹ Based as often as not on the results of mistakes as on expectations justified.

² Reliance on which together with too great an eagerness to get on with nutrition research before the stock was stabilised produced effects in the animal house at Spital which occupied much of the time of the successor of one of us in eliminating.

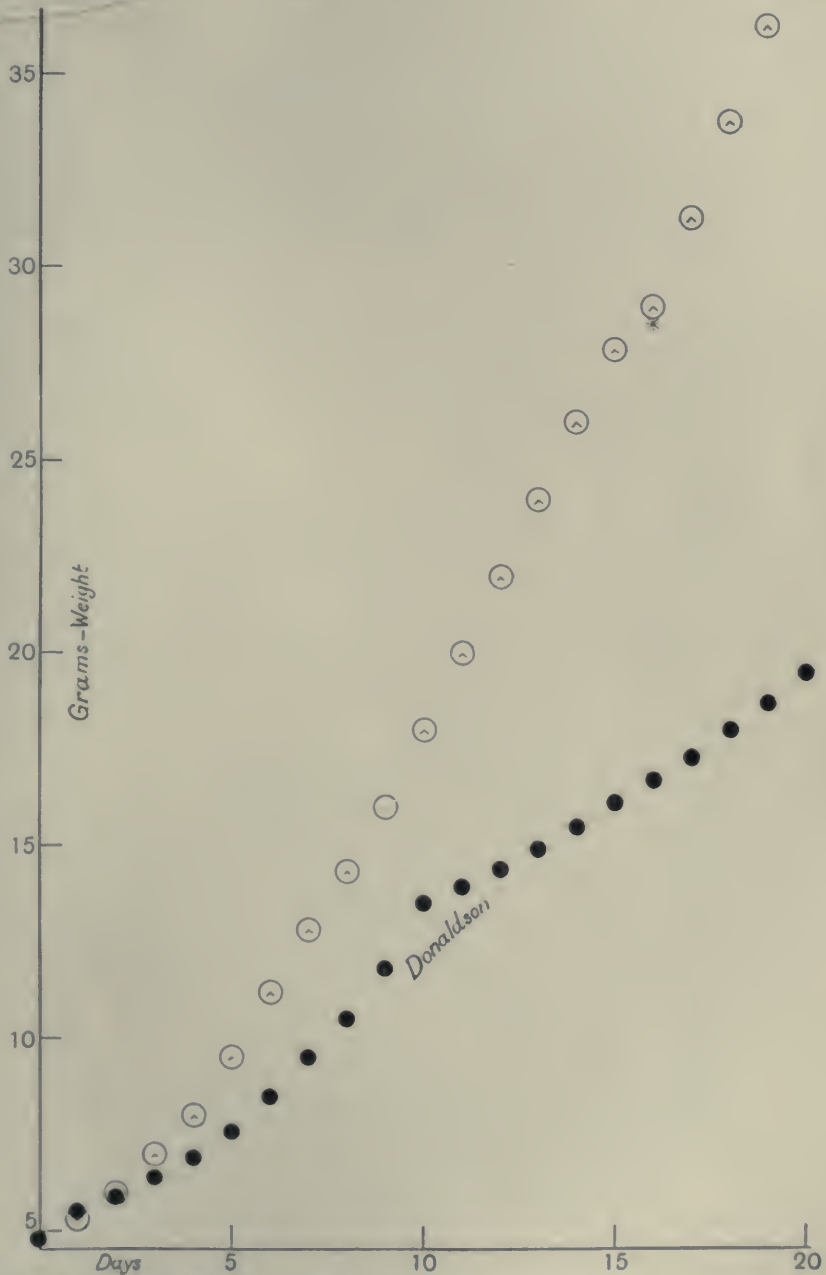


Fig. 1. Growth curves of suckling rats.

Open circles represent daily weight of rats bred by methods given in this paper.

Closed circles represent daily weight of rats as given by Donaldson.

* Animals began eating for themselves.

stock from dealers is often infected with disease which does not show up early in life and is almost impossible to eradicate. Long experience in our present laboratory (K. C. W.) backed by the similar experience of Dr C. E. Dukes, to whom we owe some of our stock, convinces us that the best way to proceed, is to begin by breeding from one or more pairs of adult animals, which have been obtained from a good source (*e.g.* another researcher) when quite young and kept on a good diet under strict observation. *Further animals should be obtained by inbreeding.* There is no evidence that inbreeding is of any danger

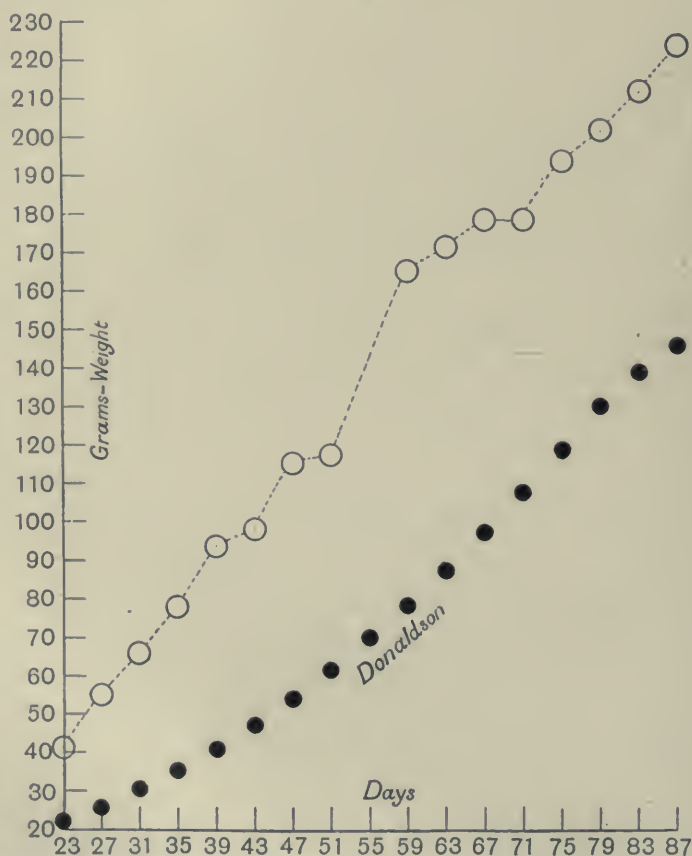


Fig. 2. Growth curves of male rats.

Closed circles are weights taken from Donaldson's book.

Open circles are weights of rats bred by methods given in this paper.

when the original stock is good. In fact inbreeding is of advantage in stabilising the stock and cutting down variations. We should be diffident in putting forward the value of inbreeding so prominently, were it not for the experience of the Wistar Institute lasting over several years, which confirms us in our impressions.

Coat colours. For preference we use black and white piebald rats as being most certain in temper and best in rearing young. Pure albinos rank next. They are less certain in temper and are not so good in rearing their first litter. When they are good mothers they are very good indeed. Other coloured rats are much more uncertain, the greys most of all. With black and white piebald rats brought up from birth or weaning by hand, so as to be used to handling, it is very rarely that one gets bitten. Whites sometimes bite when they have a litter, but much depends on how they have been handled in the past. Irish *a* and *b* rats frequently bite and the grey-piebald and self coloured have to be handled with care.

Coat colours influence the gross metabolism of the animal. In hot humid weather the whites do better than piebald black and white, and these, in turn, better than Irish *aa* and *bb* and self blacks. Such weather affects the growth and the tempers of rats very adversely. Fortunately it is not common in England.

In choice of coat colours it is well to eschew greys, self colours, Irish animals and fancy colourings and to depend on black and white piebalds or on albinos. The black and white piebalds are easier to handle and are more certain; the albinos stand heat, especially humid heat, better.

It is of advantage in experimentation to have animals variously marked. For instance in feeding experiments it is common to place pairs of the same sex in a cage together, for they like company. To know them apart it is convenient to have one albino and one piebald together, otherwise we must distinguish them by some artificial mark—ear piercing, tail clipping or what not. One of us used a simple Mendelian trick for getting equal numbers of piebald and albino rats. “Impure” piebald rats are crossed with albinos, with the result that 50 % of the offspring are albinos and 50 % piebald.

Housing. Probably most of the failure in rat breeding is due to housing. The first thing of importance is *constancy of temperature of the surroundings*. We try to keep the mean temperature 65° F. with no greater variation on either side than 5° F. Consequently we prevent, if possible, the temperature from rising above 70° F. or falling below 60° F. The animals will not breed at a low temperature. High temperature affects them adversely. Disease, especially pneumonia, is common if the temperature be not regulated. A north lighted room without great window space enables the temperature to be kept more constant. American paraffin oil stoves have been used to supplement the radiators at night with no observable detriment to the rats. Whether the duller light of a north-lighted room is an advantage or not we are unable to say. Many consider that the rat when normally fed does better in the dark than in the light. The problem of caging the animals is not difficult. Undoubtedly the animals prefer wood. It is warmer and more comfortable. But it has drawbacks in that it is more difficult to keep clean and it harbours parasites. Moreover rats are destructive and often gnaw wooden cages to pieces. Parasites, however, particularly that of scab, are easily dealt with as

described below. Some people believe that rats cannot live in metal cages. This is not true. One of us has reared generation after generation from birth to adult life in metal cages.

The present practice (G. A. H.) is to keep the pregnant and lactating does in separate individual wooden cages, $9'' \times 9'' \times 9''$, till it is time (21 days after birth) for weaning the young. Adult animals are kept in metal cages $2' 0'' \times 1' 6'' \times 1' 6''$. Not more than twenty are allowed in each cage, with three or four bucks to twelve or sixteen does. The growing animals are placed in proportionately smaller metal cages of smaller mesh. For the very young, square, and not oblong, wire meshing is used for the rats can climb on it and take exercise. A photograph of the latest design, with a platform to serve as "bedroom" is given (Fig. 3). For metabolism experiments the old round metal rat cage, opening at the side, has been discarded, for a square one on legs opening from above.

Disease. Some diseases among rats are regarded as inevitable by most researchers. Scab is an example. This disease is due to a parasite which burrows under the skin of the ears and tail. It may, unfortunately, be transmitted from animal to animal via wood. Infected shavings, wood wool or wooden cages hand on the disease. On this account one of us (V. H. M.) completely gave up the use of wooden cages and burned any shavings, etc. which had come in contact with infected animals. But we have found that wooden cages can be used and yet the disease stamped out.

The disease is easy to cure. The ears and tail of an infected animal are carefully anointed with the following mixture:

Spirit of tar	$1\frac{1}{2}$ parts
Olive oil	8 ,,
Paraffin oil	1 part

Add flowers of sulphur till the ointment is thick.

Instead of spirit of tar, creosote, in much smaller amounts, may be used and the other constituents may be varied in proportion with little harm.

Two days of application of this ointment should eliminate the disease from the animals. From the wooden cages we have got rid of the parasite by painting them within and without with a thin alcoholic solution of tar. Plenty of time was given for the alcohol to soak into the wood. Cages, disinfected thus, never harbour the parasite. In spite of severe infection prior to September 1920 when the trouble was first seriously tackled, no scab is



Fig. 3. Cage for adult rats $2' \times 1\frac{1}{2}' \times 1\frac{1}{2}'$. A. Tray in which the cage stands and forming its bottom. B. B. Palisade to prevent scattering of food and sawdust. C. Platform or "bedroom" on which the rats sleep. D. Drinking trough. E. Roof of cage, both sides shown open. The cage was made to our design by Messrs Baird and Tatlock (London), Ltd.

ever now seen, nor has it in the past ever recrudesced. Approximately 600 litters have been reared in wooden cages and no case of scab has been seen. *Scab is easily avoidable; in a well run animal house it should never occur.*

Mange, when it has occurred, has been treated with the same mixture successfully. Stock from animal dealers is likely to be mangy as well as scabby. Cure has been effected in a few days by the use of the ointment and the disease has not recurred. A loss of hair, which might be taken for mange, but is connected with diet, has been observed by one of us (G. A. H.) and will be reported on later. The common parasite, the louse, seen on elderly and sick animals, is best treated with flowers of sulphur.

An infection of the urogenital tract in does has been seen by two of us in the past in animals bought direct from a dealer. It causes a great deal of trouble. Attempts on our part, and on the part of the successor of one of us (Mr A. D. Stammers), to stamp it out, were unsuccessful. As none of the original stock, from which our present animals have been bred, suffered in that way, the disease is never seen. We should advise that any animals of unknown origin imported into an animal house, should be kept in pairs in quarantine until they produce litters, and that the litters be quarantined till they reach puberty.

Internal ear disease is sometimes seen. The animal holds its head on one side and later often starts circus movements. Such trouble seems irremediable. No correlation of it with inbreeding has been observed. It used to occur when the utmost pains were taken to secure cross-breeding. It occurs no more frequently now that the animals are intensively inbred. About twenty cases have occurred, in the last two years, in a colony which has never fallen below 300, and in summer is much larger.

Pneumonia is frequently seen. In fact it is the great danger in the rat house. The main factors affecting its incidence are temperature of the surroundings and diet. Since the surroundings of our animals have been made more equable in temperature and free from draughts; since the animals have had good food, pneumonia has greatly decreased. Outbreaks are usually referable to some unforeseen drop in temperature or a period when the "kitchen scraps" upon which the stock are fed contained too little protein.

The fact that diet causes a very appreciable difference in the frequency of the disease, makes it almost inevitable that during long-continued experiments on deficient diets, pneumonia will occur. Consequently, when possible, animals undergoing a long feeding experiment should be housed in quarters different from those of the stock. (This problem has not arisen in our present animal room, in any acute form, as the majority of the experiments have lasted three weeks only, and the diet has been adequate for every purpose except lactation.)

On the whole it does not appear to us worth while to quarantine any sick animal and try to nurse it back to health. Some vigorous, well-grown animals may recover from an incipient catarrh when quarantined and put on a good

diet. But usually the experiment does not succeed. The animal may recover temporarily, but usually succumbs again when returned to the stock cages. (This opinion must not, of course, be taken to apply to scab which can so easily and permanently be stamped out.)

Breeding. The does reach puberty at about the 90th day. On special diet they may reach it earlier. But it is not considered wise to allow impregnation till about the 6th month, when they have attained their adult stature. The present plan is then to place 16 females in a large cage with 4 well grown males. Other signs besides an increase in the size of the abdomen may help the inexperienced to judge of pregnancy, such as rapid increase in weight, uncertainty of temper, squeaking on handling, tetanic movements of fore-feet, and nest building of chance chips and straws. Any of the signs, but not all, may fail. The last two occur very shortly before the birth of the young. Experience is the best guide in judging the stage of pregnancy.

The pregnant does are removed to separate quarters, wooden boxes 9" \times 9" \times 9", ventilated in front with perforated zinc and opening with a wooden lid from above, when pregnancy is well advanced. If the does have been used to being handled from early days (birth or weaning) by a person whom they get to know, there is no difficulty in that person's handling them when they are pregnant or after the birth of the litter. Nor should the handling of the litter result in loss. Ordinarily, mothers are apt to kill and eat their young if disturbed within ten days after parturition. But when they are used to being handled by any one person from weaning, they do not resent that person's handling them or their young. Experiments which one of us (G. A. H.) has been conducting now for some years, have necessitated the daily weighing of mothers and litter from birth. Over 600 litters have been handled and it has been rare indeed to have a casualty. The main breeding seasons are spring and autumn.

The young are usually eating for themselves by the 18th day and can readily be weaned on the 21st day. The best all-round diet for the mother while the young are dependent on her, is bread and milk. No such curves of growth and no such general fitness have been seen on other diets except bread, "food casein" and egg yolk; bread, "food casein" and whey; and bread, "food casein" and excess marmite.

After weaning, the general practice is to put twenty youngsters, with perhaps one adult male as foster parent, together in one of the cages with smaller squared mesh, promoting them to larger cages as they grow. The sexes are separated early. The diet given is bread and milk supplemented with kitchen scraps. Care has to be taken with the kitchen scraps lest they contain too high a percentage of vegetable material and not enough protein and fat. The best kitchen refuse for feeding purposes contains meat, milk puddings and fish. If it is noticed that the quality of the kitchen scraps is poor, it is usual to supplement it with fresh herrings or some cheap source of animal protein and fat on the market. Animals fed on this diet grow large

and healthy and produce large litters. Adult does under 200 g. rarely occur (when they do, they are used for histological purposes) and animals of 300 g. are frequent. The males sometimes weigh 400 g.

A final point is that personal supervision every day by the researcher who is using the animals is almost essential. However good paid assistants are, it has been our repeated experience that animals do better when the person in control of the experimental work sees them and works in the animal room every day. Daily supervision, with an inspection of every animal in the animal and experimental rooms at least twice a week, should be the aim of anyone who wants good animal material upon which to work.

Summarising our experience we would say that good rat stock may be best obtained by

- (i) housing at an equable temperature of 65° F. with no more than 5° variation in either direction;
- (ii) feeding on diets containing bread and milk and kitchen scraps with a high percentage of protein and fat;
- (iii) inbreeding from originally good stock using does of six months old;
- (iv) feeding the mothers during lactation with bread and milk;
- (v) inspection and handling of stock from birth.

Thanks are due to the Medical Research Council for grants defraying the cost of the majority of the experiments in preparation for which the above experience has been gained and to Messrs Lever Bros. Ltd. for permission to publish anything bearing on work done for them.

XXVII. OXIDISING ENZYMES. VI. A NOTE ON TYROSINASE.

By MURIEL WHELDALE ONSLOW.

(Received February 26th, 1923.)

IN several previous communications [Onslow, M. W., 1920, 1921], evidence has been given in support of the hypothesis that many plants contain enzymes, termed oxygenases, which catalyse the autoxidation of aromatic compounds having two hydroxyl groups in the ortho position, with the formation of peroxides: the latter are then acted upon by peroxidases and produce "active" oxygen. The complete system, *i.e.* oxygenase, aromatic compound and peroxidase, constitutes an oxidase (phenolase or laccase).

There is another oxidising enzyme, termed tyrosinase, which has been recognised in both the animal and vegetable kingdoms. Two characteristic reactions of tyrosinase are the oxidation of tyrosine with the production, finally, of a black pigment (melanin), and the oxidation of *p*-cresol with the formation of a deep orange colour.

In regard to tyrosinase, Bach [1914] holds the view that it consists of two enzymes: a deaminase which brings about the deamination of the amino group of tyrosine with the production of *p*-hydroxyphenylacetaldehyde, carbon dioxide and ammonia, and an oxidase (phenolase) which oxidises the molecule giving the black pigment (melanin).

The purpose of the present note is to introduce the following new observations and suggestions. After summary statement, they will be considered in more detail.

1. All plants examined which give the tyrosinase reaction also contain oxidase.

2. It is suggested (in corroboration and extension of Bach's hypothesis) that tyrosinase is a mixture of enzymes, *i.e.* a water-splitting enzyme (reductase or deaminase), a carboxylase and an oxidase (consisting of oxygenase, aromatic substance and peroxidase).

3. In order to account for the action, on tyrosine, of the tyrosinase system from which the aromatic component of the oxidase has been removed by washing with alcohol, it is suggested that tyrosine itself may supply a substrate in the following way. Traces of dihydroxyphenylalanine may be formed from tyrosine in the presence of traces of "active" oxygen. Dihydroxyphenylalanine would then act as a substrate for the oxygenase, constituting, in conjunction with peroxidase, a system for producing "active" oxygen, and the reaction would be continuous. "Active" oxygen is also accepted by the

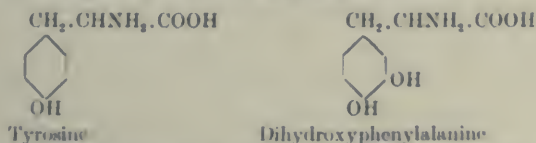
hydrogen set free by the reductase, and hence the products of reaction do not blue guaiacum.

4. The *p*-cresol reaction in plants appears to be due to oxidase alone. It is suggested that an additional hydroxyl group is introduced, as in the case of tyrosine, giving rise to an *ortho*-dihydroxy-compound which provides a substrate for the activity of the oxygenase. In the presence of peroxidase, "active" oxygen is formed and accumulates, and hence the products of the reaction blue guaiacum.

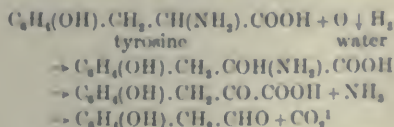
5. The instability of oxygenase is a cause of loss of activity of tyrosinase. The activity can be restored to a certain extent by hydrogen peroxide.

Now, to consider some of the above points in more detail. Some 21 plants (representing 15 Natural Orders) examined have been found to contain tyrosinase. All, in addition, contained the oxidase system previously described [Onslow, M. W., 1920]. In fact, no tyrosinase plant has yet been found which does not contain oxidase, and it appears probable that oxidase is a constant component of tyrosinase in the plant. The enzyme extract used for demonstrating the presence of tyrosinase was made exactly as for oxidase, i.e. by quickly and thoroughly pounding the tissues with 98 % alcohol and repeatedly filtering. The residue, extracted with water, gives the enzyme extract.

This extract, though practically free from the aromatic component of the oxidase, yet slowly blackens tyrosine. If an oxidase is essential to the action of tyrosinase, it would appear that, though the aromatic constituent is absent, the oxidase still functions in this case. Since it is almost impossible to obtain an enzyme preparation as above which does not give a slight blue colour with guaiacum tincture, a trace of "active" oxygen is consequently always present in the extract. The suggestion is, therefore, that in the presence of these traces of active oxygen, a second hydroxyl group is introduced into tyrosine with the resultant formation of dihydroxyphenylalanine:

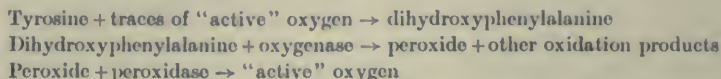


Even if such a compound were only present in traces, it would immediately act as the substrate for the oxygenase, and supplies of peroxide and hence "active" oxygen would be produced; that is, the reaction would be continuous. Further, in confirmation of Bach's view, the "active" oxygen thus produced may also act as an acceptor for the hydrogen produced by the reductase: thus the first process might be represented:



¹ Probably catalysed by carboxylase.

and the second process:



"Active" oxygen should not accumulate when the whole reaction takes place, and this is borne out by the fact that the products of the action of tyrosinase on tyrosine do not blue guaiacum. The complete process of oxidation of the tyrosine molecule involving melanin formation is doubtless complex, since the ammonia and the aldehyde are eventually involved.

Further evidence in support of the above suggestion is the statement, made by Dakin [1922], that hydrogen peroxide is able to oxidise monophenols to diphenols.

Bach has been unable to prepare tyrosinase free from oxidase: if free from oxidase, hydrogen peroxide is necessary and must be added to obtain a positive reaction (melanin formation). Peroxidase is always essential.

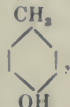
In this connexion Bach, moreover, has shown that very dilute hydrogen peroxide much increases the activity of tyrosinase itself, and may, as mentioned above, replace to a certain extent, the oxygenase-peroxide component of tyrosinase. Thus, it would assist the activity of the oxygenase, if this is present, by forming dihydroxyphenylalanine, or it might, as Bach suggests, supply active oxygen for the whole reaction for a time.

Unlike Bach, Chodat [Chodat and Wyss, 1922] maintains that he has prepared tyrosinase free from oxidase (laccase, phenolase) but, as he tested with guaiacum, he might quite well have no, or very little, reaction since his enzyme, on account of the mode of preparation, would contain only oxygenase and peroxidase and no substrate for the former. He also claims to have a preparation of tyrosinase, from one source, free from peroxidase but does not definitely state how many tests he employed to detect peroxidase.

Dihydroxyphenylalanine, itself, occurs in the vegetable kingdom, being found, for instance, throughout the tissues of the Broad Bean (*Vicia Faba*) [Guggenheim, 1913]. The plant, on injury, turns jet black owing to the oxidation of this substance. Dihydroxyphenylalanine has also been suggested as a chromogen which gives rise, through the activity of tyrosinase, to melanin pigment in animals. A solution of the crystalline product prepared from *Vicia* rapidly turns red and then black when acted upon by tyrosinase, but the products do not blue guaiacum.

The activity of the oxygenase is an important element in the whole process of tyrosinase action. If the oxygenase is impaired, as it is very readily by alcohol, heat, etc., the tyrosinase reaction is either weakened or disappears entirely, since there is no mechanism for bringing about the constant supply of "active" oxygen essential for the complete continuance of the reaction. Moreover, oxygenase is separated from peroxidase, more or less, by fractional precipitation with alcohol [Onslow, M. W., 1920], and this may well explain the deterioration of tyrosinase preparations on reprecipitation by alcohol and

the failure to purify the enzyme by this method. The oxygenase of the Basidiomycetes is said to be more resistant; hence tyrosinase from this source is more readily prepared [Bach, 1914].

Now, to turn to the *p*-cresol reaction. This substance, as in the case of tyrosine, has one hydroxyl group in the *para*-position, , and might, in

the same way, be converted into an *ortho*-dihydroxy-derivative by "active" oxygen; or traces of a dihydroxy-derivative may even be present in a solution of the substance itself. The *p*-cresol reaction has been found to be given not only by extracts (prepared as above) from tyrosinase plants, but also by extracts from all oxidase plants examined. Thus it appears to be due to oxidase, and traces of a dihydroxy-derivative from *p*-cresol may act as a substrate for the oxygenase. In the case of *p*-cresol, however, there is no reducing action, as there is no amino group. Hence "active" oxygen should accumulate, and this is so, since the products of the reaction blue guaiacum.

Apparently it is only with substances having the hydroxyl in the *para*-position that this suggested phenomenon of oxidation to a diphenol can take place. No other phenol, except catechol, as shown in earlier papers [Onslow, M. W., 1920], can act as a substrate for the oxygenase in the same way.

(Note added April 9th, 1923.) Professor Chodat has very kindly sent me a sample of tyrosinase from *Russula*, giving, on his authority, a slight reaction for peroxidase (guaiacum and hydrogen peroxide). This I confirmed: the sample gave, in addition, a powerful oxygenase reaction (catechol and guaiacum).

In conclusion, my thanks are due to Dr F. F. Blackman, F.R.S. for kindly making criticisms and suggestions.

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XXVIII. ÉTUDE DE L'ALIMENTATION ARTIFICIELLE CHEZ LE PIGEON ET DE LA DÉFICIENCE EN VITAMINES.

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Directeur: Mr le Professeur F. MALENGREAU.

(Received March 3rd, 1923.)

LES essais d'alimentation artificielle des pigeons que nous poursuivons depuis plus d'un an dans le but d'étudier les déficiences en vitamines, nous permettent de compléter aujourd'hui les données de notre premier mémoire sur ce sujet [Hoet, 1922].

Le régime de nos animaux, resté presque identique à celui dont nous avons décrit la préparation, est composé de: caséine 18, amidon de riz 60, mélange de sels de McCollum 4, papier filtre 3, margarine 10, beurre 5 parties.

Ce régime de fond est déficient en vitamine B. Pour le rendre complet et parer à cette déficience, on y ajoute de la levure ou toute autre substance riche en facteurs hydrosolubles.

Caséine et amidon. La caséine et l'amidon employés sont ceux livrés par le commerce. Ces produits, comme l'a constaté Osborne, comme nous l'avons vérifié nous-même par des expériences sur des rats, sont pratiquement dépourvus de vitamine B. Nous nous en sommes tenu à la proportion de 18 % de caséine, largement suffisante pour couvrir les besoins azotés de tous nos animaux, jeunes et adultes. Mais nous avons pu, chez ces derniers, abaisser la proportion à 12 %, pendant plus de trois mois, sans qu'il en résulte aucun trouble de nutrition. Nos résultats sur les pigeons cadrent ainsi avec ceux qu'Osborne a lui-même obtenus dans ses expériences sur les rats [Osborne, 1916].

Le beurre est soigneusement lavé plusieurs fois à l'eau tiède pour en écarter toutes les substances solubles. Le papier filtre est le papier ordinaire du laboratoire.

Levure. La levure qui a servi à combler la déficience en vitamine B, est la levure séchée du commerce, qui contient encore approximativement 10 % d'humidité.

On peut l'employer comme telle, délayée dans l'eau, mais il est préférable d'en faire l'extrait aqueux. On écarte par là les protéines coagulables et on facilite l'absorption des facteurs accessoires. Nous avons, en effet, observé

que la quantité de levure nécessaire pour maintenir l'équilibre du pigeon est plus grande avec la levure entière qu'avec le produit de sa décoction. Cela tient vraisemblablement à une digestion difficile et incomplète des cellules de levure dont une partie passe dans les fèces, comme Funk [1916] l'a observé chez l'homme.

L'extrait aqueux que nous avons couramment employé est préparé de manière à permettre des mesures comparatives de son activité: 200 g. de levure sèche délayés dans deux litres d'eau environ, sont chauffés rapidement et maintenus à une vive ébullition pendant cinq minutes. Après refroidissement, on porte la masse totale à deux litres et on la jette sur un ou plusieurs filtres. On obtient un filtrat limpide, plus ou moins coloré, dont chaque centimètre cube contient ainsi l'extrait de 0.1 g. de levure. Le filtrat est évaporé au bain marie jusqu'à une concentration cinq ou dix fois plus grande. Cette méthode, courante en chimie analytique, évite les lavages toujours ennuyeux et permet d'obtenir une solution d'activité constante; elle est applicable ici, le résidu insoluble n'ayant aucun pouvoir adsorbant vis-à-vis des facteurs accessoires. On peut concentrer l'extrait davantage encore et lui enlever une partie de ses sels en les précipitant par addition d'un volume égal d'alcool à 95° qu'on chasse, après filtration, par distillation sous pression réduite.

Au lieu d'incorporer l'extrait de levure à la pâte alimentaire comme on le fait généralement pour les rats, nous préférons le donner séparément à intervalles réguliers de un, deux ou trois jours. La quantité que reçoit le pigeon, est ainsi exactement mesurée et on évite les influences psychiques que peut avoir sur l'appétit de l'animal toute modification de saveur ou d'odeur de la nourriture.

Influence des vitamines B sur l'appétit des pigeons.

Nous avons montré dans notre premier mémoire comment le régime rendu déficient par suppression de la levure, détermine aussitôt chez le pigeon une diminution d'appétit avec perte progressive de poids aboutissant après un nombre variable de 8 à 20 jours aux manifestations convulsives de la polynévrite aviaire. Cette diminution d'appétit est de règle chez les rats où Funk l'avait signalée dès 1914 [Funk, 1915]. Mais à l'encontre de ce qui se passe chez ces derniers où l'anorexie est tardive et ne se répercute sur la courbe de poids qu'après deux à trois semaines de régime déficient, l'effet, chez le pigeon, est immédiat et peut prendre moyennant certaines précautions, une allure d'une grande régularité. De toutes ces précautions, la plus importante est d'avoir un pain toujours également frais, distribué à la même heure avec une eau journellement renouvelée. On peut mesurer l'appétit, soit par la quantité ingérée d'aliments, soit par les variations de la courbe pondérale. Dans les deux cas les chiffres obtenus présentent des variations comparables. Le tableau I indique les pesées de trois pigeons observés pendant une période de quinze jours. Tous les trois reçoivent du régime déficient *ad libitum*. On leur donne en plus tous les quatre jours le quadruple ou un peu moins de la dose

journalière minima de levure, ce qui a pour résultat de les faire manger, gloutonnement pendant la première journée, puis de moins en moins pendant les jours qui suivent; leur courbe de poids affecte ainsi l'aspect d'une ligne brisée presque régulière avec des hauts et bas coïncidant respectivement au jour le plus proche et le plus éloigné de l'ingestion de levure.

Tableau I.

Dates juillet	Poids des pigeons			Quantité donnée de levure (sous forme d'extrait)
	No. 19 g.	No. 31 g.	No. 36 g.	
6	400	343	388	2.4
7	370	323	365	—
8	360	312	350	—
9	348	310	336	—
10	370	317	364	2.4
11	377	312	380	—
12	358	308	349	—
13	351	304	349	—
14	382	312	350	3.0
15	365	334	372	—
16	360	320	361	—
17	362	314	355	—
18	373	318	366	3.0
19	354	315	358	—
20	359	320	355	—
21	348	313	346	—

Sans obtenir toujours une régularité aussi grande, il est incontestable qu'un des effets immédiats de la vitamine B est de stimuler fortement l'appétit, et cela, dans des conditions où l'on peut exclure toute influence psychique pouvant provenir de la saveur particulière des aliments.

Il serait inexact cependant, et plus personne n'y songe aujourd'hui, de considérer la vitamine B comme un simple apéritif et de confondre l'avitaminose avec l'inanition; la quantité de nourriture ingérée diminue mais ne tombe jamais à zéro. Les études de Terroine et Barthélemy [1922, 1, 2] sur les manifestations comparées de l'inanition et de la déficience sont d'ailleurs décisives à cet égard.

DOSE MINIMA DE LEVURE.

La mesure de la dose minima de levure nécessaire pour prévenir les manifestations de la déficience n'est pas toujours facile. On connaît la discordance entre les chiffres mis en avant par les divers auteurs à propos de la quantité requise pour la croissance des jeunes rats. Outre les différences individuelles qu'on relève d'un animal à l'autre, il faut tenir compte de ce que les levures ont une richesse en vitamine B variable d'après la nature du moût sur lequel elles se sont développées. Tout récemment encore Kennedy et Palmer [1922] mettaient en doute la valeur de la levure comme source de vitamine B, dans les expériences de nutrition sur les rats à cause de sa grande irrégularité. Nos résultats ne sont donc valables que pour les conditions où nous avons opéré.

Chez les pigeons, d'une façon générale, quels que soient l'âge et le sexe,

les différences individuelles nous ont paru moins grandes que chez les rats. L'extrait de 1 g. de notre levure sèche a toujours été suffisant, soit pour prévenir la polynévrite avec maintien du poids normal, soit pour la guérir avec retour rapide au poids initial. La dose minima oscille habituellement entre 0.80 et 0.90 g. pour des pigeons de 350 à 400 g.; elle semble fléchir quand diminue le poids de l'animal.

Chez les pigeons de 250 à 300 g., en pleine déficience, avec phénomènes convulsifs, une dose quotidienne d'extrait de 0.60 g. de levure suffit pour supprimer en quelques heures les manifestations nerveuses et provoquer une augmentation de poids, mais le relèvement de la courbe pondérale est lent et n'atteint jamais le niveau normal.

De l'ensemble de nos recherches sur l'alimentation artificielle des pigeons, nous sommes en mesure aujourd'hui de conclure que notre mélange d'aliments purifiés, quand il est complété par de l'extrait de levure comme source de vitamine B, répond à toutes les exigences d'une alimentation complète: il maintient sans altération aucune l'équilibre physiologique de nos animaux, assure, comme nous allons le voir, la fonction de reproduction et permet la croissance des petits. Au cours de 18 mois d'essais, une soixantaine de pigeons ont été nourris exclusivement avec ce mélange, *que tous mangent spontanément*, sans que nous ayons observé un seul cas de dégoût ou d'intolérance même passagère. Le tableau II donne quelques renseignements sur les pigeons actuellement en expérience et qui n'ont jamais cessé d'être au régime artificiel exclusif malgré des déficiences plusieurs fois provoquées.

Tableau II.

Numéros des pigeons	Régime artificiel		Poids des pigeons		Nombre de crises de déficience	Observations
	Date du début	Durée actuelle (mois)	Au début g.	Au 20. ii. 23 g.		
2	16. ix. 21	17	390	415	6	
3	14. x. 21	16	430	430	5	
10	10. xii. 21	14	450	420	5	
12	14. xii. 21	14	465	400	6	
22	12. xi. 21	14	520	435	4	
25	10. ii. 22	12	430	370	2	
26	10. ii. 22	12	455	465	1	
27	10. ii. 22	12	365	355	1	A pondu 6 œufs
39	16. iii. 22	11	390	392	2	
40	16. iii. 22	11	375	387	2	
50	1. iv. 22	10	290	370	1	
51	1. iv. 22	10	252	410	1	
52	1. iv. 22	10	275	420	1	Jeunes pigeons pris à l'âge de 4 à 5 semaines
53	1. iv. 22	10	280	383	1	
60	15. viii. 22	6	310	400	1	
63	15. viii. 22	6	284	430	1	

Le régime artificiel permet ainsi des études sur l'alimentation des pigeons superposables à celles qui se font sur les rats. Là comme ici, une alimentation

pour être complète, c'est-à-dire pour répondre à tous les besoins de l'organisme, devra assurer pendant une longue période l'équilibre physiologique de l'animal. La condition est formelle: une substance ne sera active comme vitamine B que si elle a une action à la fois préventive et curative autant sur la courbe de poids que sur les accidents nerveux de la polynévrite aviaire. Le poids des animaux, à la fin d'une expérience, devra donc s'être maintenu ou être revenu au niveau initial et, dans ce dernier cas, s'y maintenir encore pendant deux ou trois semaines au moins par la seule addition de la substance au régime déficient. Ces conditions ont toujours été exigées dans les recherches sur l'alimentation chez les rats, et il est étonnant qu'on en ait fait si peu de cas dans les expériences sur les pigeons.

Le fait d'empêcher la crise de se produire endéans les 50 jours, mais avec des pertes de poids allant de 20 à 30 % n'est pas un critère suffisant d'une action préventive. Autant considérer comme satisfaisante une alimentation qui, si elle était continuée plus longtemps, aboutirait finalement au dépérissement et à la mort.

Les erreurs commises dans l'étude de la vitamine B tiennent à deux causes: à l'absence de régime artificiel pour pigeons et à l'importance exagérée qu'on a trop longtemps attribuée aux phénomènes nerveux de la crise polynévritique. Ceux-ci ne sont nullement pathognomoniques de la déficience en vitamine B: ils relèvent de causes multiples et variées et cèdent à des substances qui n'ont entre elles aucun lien ni chimique ni biologique. Tout récemment, Koskowsky [1922] a montré que des injections d'histamine chez des pigeons en déficience préviennent la crise sans empêcher la baisse de poids ni la mort. La tyramine [Lipschitz, 1923] se comporte de même. Ces substances, il y a quelques années encore, auraient passé pour vitamines antinévritiques. En réalité leur pouvoir curatif sur les phénomènes convulsifs n'est pas à confondre avec l'action des facteurs accessoires.

Le régime artificiel permet l'étude comparative de l'alimentation chez le pigeon et chez le rat; c'est ainsi que du seul point de vue des facteurs hydro-solubles et en admettant l'hypothèse possible mais non encore démontrée, que la vitamine antinévritique est identique aux facteurs B de croissance, nous voyons le pigeon particulièrement sensible à cette sorte de déficience. Extrêmement tardive chez le rat adulte, n'arrêtant la croissance des petits qu'après deux ou trois semaines, la déficience a chez les pigeons au contraire un effet immédiat qui permet en quelques jours de la déceler. Aussi croyons-nous ces animaux appelés à rendre de grands services encore dans l'étude des vitamines.

REPRODUCTION ET CROISSANCE.

Une alimentation n'est réellement complète pour un animal que si elle assure la reproduction de l'espèce et permet la bonne croissance des petits. C'est à côté de beaucoup d'autres, une raison suffisante pour rejeter, en ce qui concerne les pigeons, le régime de riz poli additionné de levure [Seidell, 1922].

Nous devons nous demander si notre régime artificiel était à même de réaliser cette condition. Etant donné les résultats obtenus chez les rats avec une alimentation pareille à la nôtre, nous pouvions l'espérer. Nos essais qui se sont prolongés pendant une période de huit mois, depuis le printemps jusqu'au milieu de l'hiver 1922, ont confirmé nos prévisions. Pendant toute cette période la ponte a été régulière chez les quatre femelles soumises au régime artificiel depuis plus de quatre mois et qui, ensemble, ont pondu 27 œufs d'apparence normale avec des jaunes bien colorés.

Malheureusement les conditions expérimentales sont fort défavorables à la couvaison. De fait, nous n'avons obtenu que deux éclosions sur 20 œufs couvés. Les autres contenaient des embryons normaux morts à des périodes variables de leur développement, parfois très rapprochées du terme. Mais un seul résultat positif a ici une valeur démonstrative que n'ont pas les essais négatifs. Les deux jeunes pigeons, soumis à leur tour au régime artificiel se sont parfaitement développés (Graphique I, n° 29 et 70). Nous avons complété ces expériences en élevant, par gavage cette fois, deux autres pigeonceaux âgés de huit jours (Graphique I, H_1 et H_2), et six autres âgés de quatre à cinq semaines (Tableau II). Tous ont eu une croissance normale et sont aujourd'hui des animaux adultes de belle apparence.

Nous étions occupé à la rédaction de ces notes quand nous est parvenu le travail de Benedict et Sugiura [1923]. Ces auteurs qui n'ont pas eu connaissance de notre premier mémoire ainsi que de celui de Simonnet [1921], ont entrepris, simultanément avec nous, des essais identiques d'alimentation artificielle chez les pigeons. Leurs conclusions en ce qui concerne l'équilibre physiologique, la reproduction et la croissance de leurs animaux, sont pareilles aux nôtres et les confirment.

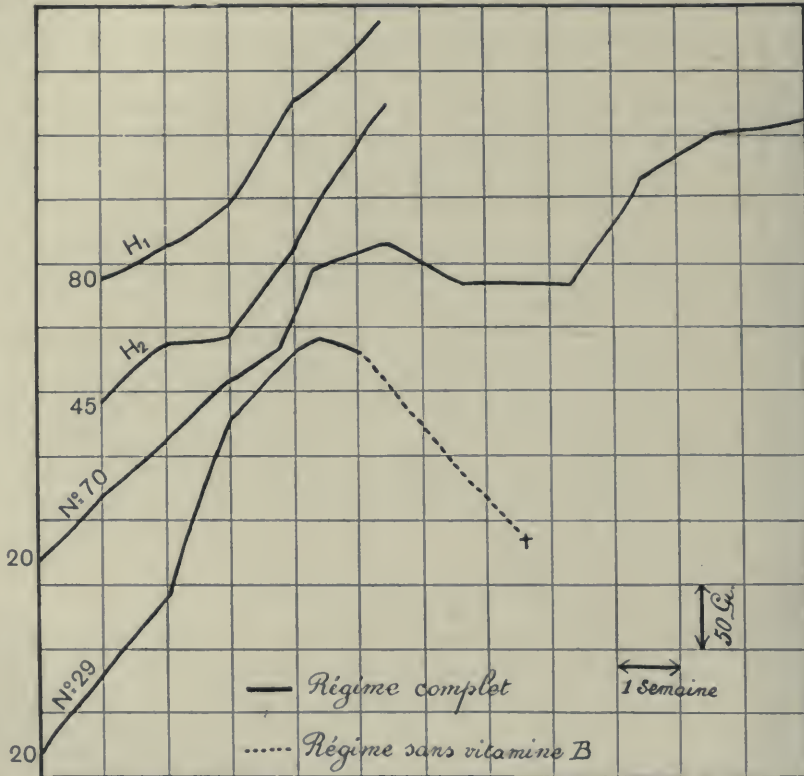
DÉFICIENCE EN VITAMINE A.

Nos expériences sur la déficience en vitamine A chez les pigeons nourris artificiellement, sont en cours depuis cinq mois. Nous aurions voulu les prolonger davantage, mais le travail de Benedict et Sugiura dont nous venons de parler et qui traite du même sujet, nous oblige à quelques remarques et à la publication, dès à présent, d'une partie de nos résultats.

L'étude des vitamines liposolubles se heurte à deux sortes de difficultés qui sont, l'une, la purification malaisée des aliments qui entrent dans la composition du régime, et l'autre, la lenteur que mettent les animaux à répondre aux déficiences en facteurs A, par opposition à ce qui se passe pour les facteurs B. Dans leur travail critique sur la question, Osborne et Mendel [1921] insistent sur ces difficultés: "... (the) removal of the fat-soluble vitamine from even purified proteins and carbohydrates is accomplished with far greater difficulty than has been hitherto suspected. An entirely convincing crucial experiment, in which nutritive failure immediately ensues upon the administration of diets fully adequate in every respect except for the presence of fat-soluble vitamine, remains to be made. It is significant that older rats thrive

for a longer time than the younger ones on the same diets, nearly, if not entirely, free from the fat-soluble vitamine. This is in contrast with the well established fact that at all periods the lack of water-soluble vitamine is speedily manifested." Ils disent également en parlant de la vitamine A: "...some, like ourselves, have described their animals as usually growing at a nearly, or quite, normal rate for periods of 60 to 80 days before showing signs of a nutritive deficiency in the diet."

Sans nier l'intérêt du travail de Benedict et Sugiura ni contredire leurs résultats, il faut reconnaître cependant que leurs essais ne sont pas à l'abri de



Graphique I. Croissance de jeunes pigeons mis au régime artificiel complet dès leur naissance. Le pigeon 29 est mort au cours d'une expérience de déficience en vitamine B.

toute critique. Une de leurs conclusions est la suivante: "Pigeons on a diet of sufficient caloric value, even though the diet lacks fat and fat-soluble vitamine, may maintain excellent condition, and may produce fertile eggs and rear healthy squabs. Hence fat-soluble vitamine is not essential in any stage of avian nutrition."

Nous voulons bien admettre que les aliments qui entrent dans la composition des régimes B et C de ces auteurs, soient totalement dépourvus de vitamine A, il n'en reste pas moins que leurs expériences ont une durée qui ne dépasse pas 70 jours pour la plus longue et qui, souvent même, ne va au

délà de 30 jours quand il s'agit de la croissance des jeunes pigeons. Cette durée est manifestement insuffisante et enlève aux expériences une bonne part de leur valeur de démonstration.

Nous avons employé pour unique matière grasse l'huile d'arachide durcie par hydrogénation (hardened fat)¹, exempte de vitamine A [Committee of the Royal Society, 1919; Drummond, 1922; Report on accessory food factors, No. 38, 1919].

Notre régime avait ainsi pour composition: caséine 18, amidon 60, huile d'arachide 15, sels 4 et papier filtré 3 parties; l'extrait de levure était donné séparément à la dose de 1 g. par jour et par pigeon. Nous avons tenu néanmoins à vérifier encore la déficience en vitamine A du mélange par un essai sur quatre jeunes rats: leur croissance s'est arrêtée après quelques semaines et leur poids a diminué progressivement. La xérophthalmie est apparue au troisième mois chez l'un d'eux, qui est mort peu après. Chez un second les manifestations oculaires se sont déclarées après trois mois et demi.

Depuis le 27 septembre 1922 ce régime est donné *ad libitum* à six pigeons (les n^{os} 51, 52, 53, 50, 26 et 27), tous les six au régime artificiel complet depuis plus d'une demie année. L'expérience dure maintenant depuis cinq mois et rien jusqu'à présent, ni dans l'aspect général, ni dans le plumage, ni dans la température, ni dans le poids de nos animaux ne traduit le moindre trouble. Bien mieux, une femelle vient de pondre deux œufs et les couve actuellement. Nous donnons ci-dessous la courbe pondérale de ces six pigeons (Graphique II). Ces courbes présentent vers la quatrième semaine du régime déficient en facteurs A un fléchissement prononcé suivi, 15 jours après, d'un relèvement rapide, dû à une insuffisance, involontaire et momentanée de levure². Cet accident que nous n'avions pas cherché, montre que les pigeons sans vitamine A se comportent vis-à-vis d'une déficience juxtaposée de vitamine B, comme des pigeons normaux.

Les expériences, bien qu'inachevées, confirment partiellement les conclusions de Benedict et Sugiura et montrent l'inutilité, au moins pour le pigeon adulte, des facteurs liposolubles.

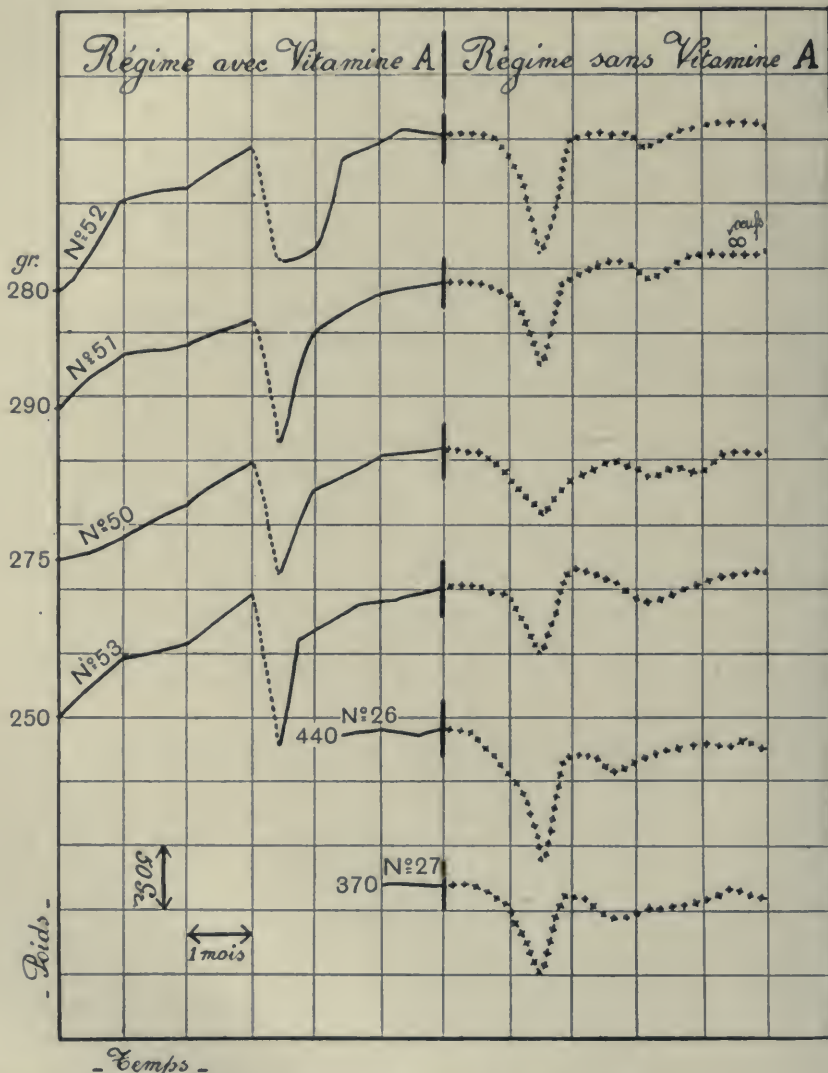
Ces résultats peuvent paraître étranges si on les compare à ceux qu'on obtient avec des poussins dans des conditions presque identiques. Hart, Halpin et Steenbock [1922] dans leur étude du "leg-weakness," signalent en effet l'importance de l'huile de foie de morue pour ceux qu'on nourrit artificiellement. Emmett et Peacock [1922] décrivent de leur côté toute une symptomatologie de l'avitaminose à chez les jeunes poules: anémie, œdème, mauvais état des plumes, début de xérophthalmie, etc.... Plimmer et Rosedale

¹ Cette huile a été mise gracieusement à notre disposition par la firme "Jurgens" de Zwynndrecht. Nous sommes heureux de leur en exprimer ici notre vive reconnaissance.

² Cette insuffisance était due à ce que la dose quotidienne de un gramme de levure, au lieu d'être donnée sous forme d'extrait, l'avait été pendant les six premières semaines sous forme de levure entière délayée dans la pâte. Lorsque nous eûmes substitué l'extrait à la levure comme telle, les symptômes de déficience disparurent immédiatement. Tous les autres pigeons du laboratoire ont simultanément et pour la même raison, présenté les mêmes phénomènes (voir p. 221).

[1922] et d'autres [Nelson, Lamb and Heller, 1922] concluent, eux aussi, à la nécessité de la vitamine liposoluble dans leur alimentation.

De l'avis de tous, l'élevage des poussins est difficile et nous même l'avons tenté, sans succès, au moyen de notre régime artificiel. Aussi, sans nier la possibilité d'action sur eux de vitamines inutiles aux pigeons, nous croyons



Graphique II. Le trait plein ——— représente le régime complet.
 „ pointillé - - - - - „ „ sans vitamine B.
 „ en croix + + + + „ „ „ A.

Les pigeons Nos. 50, 51, 52, 53 étaient âgés d'un mois, quand ils ont été mis au régime artificiel. La première partie de leur courbe représente leur croissance à partir de ce moment. Après trois mois, ils ont été mis pendant 15 jours au régime sans vitamine B, après sept mois définitivement au régime sans vitamine A.



N° 1. Pigeon (n° 2) après 18 mois de régime artificiel (voir tableau II).

N° 2. Pigeon (n° 70) âgé de 5 mois au régime artificiel depuis sa naissance, les parents au moment de la ponte étaient, eux aussi, au régime artificiel depuis 4 mois.

N° 3 et 4. Pigeons (nos 50 et 52) mis au régime artificiel complet à l'âge d'un mois, âgés actuellement de 11 mois, et au régime sans vitamine A depuis 5 mois.

nécessaire, avant d'admettre pareille conclusion, de s'assurer que les phénomènes nutritifs ne sont pas, en ce qui les concerne, conditionnés par des influences étrangères qui nous échappent encore.

CONCLUSIONS.

1. Une alimentation artificielle composée de caséine, d'hydrates de carbone, de graisses, de sels et de cellulose, complétée par de l'extrait de levure (0·8 à 0·9 g. minimum de levure sèche) est suffisante, en l'absence de facteur C pour maintenir l'équilibre physiologique du pigeon, assurer la reproduction et permettre la croissance des petits.

2. Le facteur A ne semble pas nécessaire au pigeon adulte.

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XXIX. ON THE THEORY OF GELS. IV.

BY SAMUEL CLEMENT BRADFORD.

From the Science Museum, London.

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THE investigation of the physical properties of the natural emulsoids has produced an enormous literature. For the correlation of this, and for the study of many problems of biology and industry a theory of the sol-gel transformation is needed which fits the facts. The present condition of uncertainty is illustrated by the multitude of mutually incompatible *ad hoc* theories of gel structure which are current. The many hypotheses may be classified in three groups: (1) one-phase or molecular systems, (2) two-phase liquid-liquid systems and (3) two-phase liquid-solid systems. To theories in the first class it must be objected that they are unable to explain the loss of mobility that occurs on setting. Those in the second group have the additional difficulty that no liquid-liquid systems can be imagined which would have the elastic properties of gels [Hatschek, 1917]. Nor is there any direct evidence for hypotheses in either of these two categories. Thus we are obliged to assume that gels have a solid phase. This idea is the most natural and was the earliest to be held [Frankenheim, 1835]. The suggestion of K. von Nägeli [1858] is well known, that gels are composed of molecular complexes or micellae with crystalline properties, in the interstices within and between which the water is held by molecular attraction. It has been inferred that von Nägeli intended a geometrical framework, and attempts have been made to devise a network that would account for the elastic and thermal properties of gels. If, however, it is realised both that the elastic properties of gels differ greatly and that the directive forces inherent in the ultimate particles of gels are probably different, it seems unlikely that a single framework could be found to account for the different properties of different gels. It is more probable that the structure of gels varies according to the nature of the gel substance. Bütschli's hypothesis [1892, 1896, 1898, 1900] that gels have a honeycomb structure is disproved by the work of Zsigmondy [1911], Anderson [1914] and Bachmann [1917] on the vapour pressure isotherms of gels, which shows that they must contain fine pores with a radius of from 2.5 to 5 μ , some 300 times smaller than Bütschli's honeycombs. These experiments are particularly interesting as they give an explanation of van Bemmelen's [1878, 1880] curious hysteresis cycle. From microscopic work on soap curds and gels, Zsigmondy and Bachmann [1912] have favoured a fibrillar structure. On the other hand McBain [1920] considers that identical colloidal particles are present in the sol and gel

state, which differ only in mechanical rigidity and elasticity. Moeller [1916, 1917] supports the view that gelatin gels have a fibrillar structure. But it must be objected to his experiments that the use of alcohol and tanning reagents may alter the structure of the gels or modify the gelation process. Barratt [1920], also, has given evidence that fibrinogen gels are composed of fibrils. For the soaps and fibrinogen there is direct microscopic evidence that fibrils can be formed by the cooling solutions, which suggests that the ultra- or amicroscopic structure of the gels may be fibrillar. For gelatin, agar and silicic acid, however, a globulitic structure is indicated. In gelating 2 % gelatin, submicrons develop gradually from 4, showing Brownian movement, in a square division of the field with a side of 9μ , to 80 to 100 at rest in the same area [Mans, 1900]. Moreover the ultramicroscopic appearance of gelatin

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(Note added on May 17th, 1923.) Since writing this paper Professor Schryver has very kindly supplied a small sample of specially pure isoelectric gelatin prepared by a new method. This readily yields dilute jellies with marked granular structure in which small spherical particles can be separately resolved, and appears, generally, to behave like the gelatins used for these experiments, except that some doubt has arisen whether large spherites can be obtained under the conditions described.

S. C. B.

subject so vast needs many workers. When these relations are understood, it may be possible to deduce a sound theory of the stability of colloid solutions.

Fortunately, however, von Weimarn's long series of experiments [1914] have thrown some light on the conditions governing the separation of solid from solution. His formula

$$N = U \frac{P}{L}$$

expresses a relation between N , the "form coefficient" of the precipitate, and U , P and L , respectively functions of the viscosity of the reaction medium together with the size and structure of the particles in solution, the excess concentration of the substance to be precipitated, and its solubility. From a great many experiments von Weimarn was able to show that, as N increases, the precipitate passes through stages in which it appears as (1) large complete crystals only after some years, (2) ordinary crystals in a short time (3) growth figures or needles, (4) amorphous precipitates frequently showing microscopic spherical grains, and (5) as a gel which cannot be resolved by the microscope. The colloid condition results when N is extremely great. If, at the same time,

the excess concentration, P , is small, a sol is produced; if P is great, a gel is formed. The formula is not exact. Experiments in progress may help to a more definite statement. But, in practice, the incomplete equation is very useful in showing how to vary the grain size of a given material. The formula suggests directly that gelation is merely an extreme case of crystallisation and that gelatin and agar are substances whose properties lead naturally to a high value of N [Bradford, 1917; 1918; 1920, 1, 2; 1921]. This hypothesis is abundantly confirmed by experiment.

Gelatin sols behave exactly as supersaturated solutions. Certain curious temperature relations of the sols can be simply explained only on the assumption that gelatin has a definite solubility at a given temperature. Moreover, by reducing the value of N in von Weimarn's formula, the particles of gelatin gels can be increased in size as desired and are found to be definitely crystalline.

The molecular theory of solution [Bradford, 1919, 1922] shows that every substance must have a definite solubility, however small, in a given solvent, with which it does not combine chemically. Consider the case of a hot solution of a substance, of high molecular weight and very low diffusivity, in a liquid, in which its solubility, L , is considerable at high temperatures but small at low.

On cooling, the Noyes-Nernst formula,

$$V = \frac{D}{\delta} S (C - L),$$

shows that the velocity of crystallisation will be small, owing to the very small value of the diffusion coefficient, D . The solution will become gradually more and more supersaturated and the particles of solute increasingly aggregated. The value of L in von Weimarn's formula will diminish rapidly, while P increases correspondingly. When, eventually, spontaneous crystallisation occurs, U will be large on account of the high molecular weight and aggregation, P also will be large and L small, giving a maximum value of N . Therefore, the solution will set slowly to a jelly.

A substance with greater solubility, such as oxy-haemoglobin, or egg albumin, will be obtained as micro- or macroscopic crystals even though its molecular weight be very high. But it can be shown that gelatin corresponds exactly to the case considered above. Its molecular weight is not known. However, Dakin's recent analyses [1920] show that gelatin contains 1.4 % of phenylalanine of molecular weight 165. Since one molecule of gelatin cannot contain less than one molecule of phenylalanine the lowest possible molecular weight of gelatin is 11,800. Loeb's [1922] osmotic pressure experiments indicate twice this figure. It is safe to conclude, therefore, that gelatin has a very high molecular weight. A value of 10,000 would correspond to molecules themselves of colloid size with a diameter of 1.75μ .

The solubility of gelatin was determined in the course of experiments to show that it behaves exactly in accordance with von Weimarn's formula. Dilute filtered solutions of ashless gelatin, purified by electrolysis, from the

Osmosis Co., of different strengths, which had been quickly boiled and sealed in tubes, were allowed to stand at room temperature. Instead of setting to a jelly the solutions became opalescent and the excess of gelatin was deposited slowly as a gelatinous precipitate. When the volumes of the precipitate were plotted against the concentrations, the curve was found to be a straight line cutting the concentration axis at the point corresponding to 0.12 % of dry gelatin per 100 cc. of solution. Above this concentration the volume of the precipitate is proportional to the excess concentration. This figure is undoubtedly the true solubility of gelatin at room temperature, which remained fairly uniform at 18° during the experiment.

Confirmation was supplied by making up solutions of 0.12, 0.13 and 0.135 % and allowing to stand as before. The first remained perfectly clear, the second developed in a few days a beautiful clear bluish opalescence which persisted for months. This was evidently a metastable solution of gelatin, the permanence of which was due to the low diffusion constant of gelatin. With the 0.135 % solution the metastable limit was overstepped and a trace of gelatinous precipitate was deposited. Further confirmation is afforded by the fact that water stirred with gelatin for two hours takes up 0.114 g. of solid. The experiments are being continued with ashless gelatin in a thermostat at different temperatures, and the solubility is being calculated, also, from the amount of solid left in solution in the mother liquor. The results leave no doubt that gelatin has a true solubility in water which increases rapidly with rise of temperature. The high molecular weight is sufficient to account for a very low value of the diffusion constant. Thus gelatin fulfils all the conditions which have been assumed in the hypothetical case considered above. It follows that von Weimarn's formula is sufficient to explain the occurrence of gelatin in the colloid state and that the gelation of gelatin sols is an extreme case of crystallisation.

The theory requires that there shall be a gradual increase in the size of the deposited solid gelatin particles with decrease in supersaturation, until, with very dilute solutions, the precipitated particles become visible microscopically. This is exactly what happens. Six per cent. gelatin gels, from which the liquid has been expressed, cannot be differentiated in the ultra-microscope [Bachmann, 1911]. With less concentration a globulitic structure becomes apparent, and the size of the granules increases as the concentration is reduced. When the concentration is reduced to 0.4 % microscopic examination of the white gelatinous precipitate gives the appearance of an enormous number of particles like grains of sand, just too small to be differentiated, among which there are a number of larger spherical particles big enough to be seen. These are mostly aggregated into clusters. With further decrease in concentration the sand-like appearance becomes more marked and the larger spherical particles fewer and bigger, until, at 0.2 % they reach nearly 1μ in size. When a gelatin solution is cooled, the bulk of the precipitate increases with the concentration and the particles decrease in size until, at about 0.7 % at 18°

the precipitate fills the solution and forms a continuous jelly. This jelly is white and opaque owing to the large size of the particles. As the concentration is increased the particles get smaller and eventually the jelly becomes clear. A gelatin jelly is, therefore, a gelatinous precipitate of gelatin of more than 0.7 % concentration. The relative sizes of the grains can be determined also in another way. Solutions of ashless gelatin, containing from 0.25 to 1.0 % of dry gelatin, were boiled and sealed in test-tubes and allowed to stand at room temperature. Within 24 hours the solutions up to 0.51 % had become distinctly opalescent, 0.77 % solutions had set to very cloudy just coherent jellies and the 1.0 % tubes contained a slightly stronger jelly that was still rather more cloudy. In about three weeks, the jellies had separated a film of clear liquid above. In the remaining tubes, containing the weaker solutions, the opalescence developed from day to day, and, after about ten days, the precipitate appeared to be subsiding in the tubes. The rate of fall was measured from time to time, and the curious fact was observed that there are three distinct stages in the fall of the surface of the opalescence: (1) a long gradual fall at a uniform rate, for from about 20 to 30 days according to the concentration, during which time the particles are discrete, because the surface of the opalescence is easily disturbed, (2) a sudden drop, for one or two days, after which the precipitate appears to have coalesced to a continuous jelly, and (3) a gradual shrinkage, at a somewhat slower rate than (1), which shows all the signs of syneresis. The application of Stokes's law to the long continuous free fall, (1), enables the relative sizes of the particles to be calculated, and confirms the fact that particles increase in size with diminishing supersaturation. These experiments are being continued in a thermostat at different temperatures, from which it is hoped to determine a more exact relation between the size of the precipitated particles and the solubility and excess concentration. However, each series takes several months, and the solubility increases rapidly with rising temperature, so that a preliminary series is necessary at each stage. Consequently, with very little opportunity for experimental work, it will be some time before the complete results can be published. Similar experiments with dialysed agar give exactly comparable results.

There is no doubt that the solubility of gelatin, like that of any other substance, varies with temperature and with the presence of other bodies. As an amphoteric compound, it is particularly sensitive to changes in the pH . This is confirmed by Loeb's experiments on the rate of solution of gelatin in the presence of salts, acids and alkalis. If, therefore, a solution of gelatin contain more of this substance than corresponds to its solubility under the conditions of the experiment, the excess will be deposited slowly. The gelatin molecules will aggregate gradually in the process of precipitation, and the properties of the liquid will be affected by the presence of these aggregates. Experiments on the viscosity of gelatin solutions compelled Loeb [1922] to assume that the forces holding gelatin and crystalloids in solution are the

same. The viscosity of gelatin solutions is essentially different from that of simple amino-acids or of egg albumin. It is of an altogether higher order, and is much affected by the presence of electrolytes. If it be assumed, as experiments indicate, that the viscosity of gelatin and albumin solutions is a function of the volume of the protein in solution, gelatin solutions must possess a mechanism, which albumin solutions do not, of increasing the volume of the solute. This mechanism is likely to be connected with their tendency to set to a jelly. From which it may be concluded that, in gelatin solutions, solid particles of gelatin, or micellae, are formed gradually, which occlude relatively large quantities of water, whereby the relative volume of the particles is increased.

In egg albumin solutions these particles are lacking because the solutions do not set, *i.e.* the solubility is greater. But if the p_H is below 1.0 and the temperature higher, solutions of albumin do tend to gelate, and then their viscosity is of the same order as that of gelatin solutions.

The influence of electrolytes on the viscosity of gelatin solutions, and not of albumin solutions, is explained by the presence of a Donnan equilibrium between the submicroscopic gelatin particles and the surrounding solution, which regulates the amount of water occluded. This was confirmed by experiments on the viscosity of suspensions of finely powdered gelatin in water, whose viscosity varied directly with the observed volume of the particles, and was influenced by electrolytes exactly as are solutions of gelatin. The effect of electrolytes on the viscosity of gelatin solutions would be explained if it could be shown that they contain submicroscopic particles of solid gelatin capable of swelling. Loeb gives much indirect evidence that solid particles are formed in gelatin solutions, under given conditions, when the temperature falls below a certain point. For instance, the viscosity of 2 % gelatin at p_H 2.7 increases rapidly on standing at temperatures below a point which lies between 25° and 35° and which the experimental values indicate to be at about 27° to 28°. Above this point the viscosity decreases slowly at rates which increase slightly with rising temperature. That is to say a 2 % solution of gelatin at p_H 2.7 is saturated at about 27° or 28°. Below this temperature the excess of gelatin gradually forms solid aggregates, and at higher temperatures the aggregates are gradually dissolved. The actual proof is supplied by direct experiments on the crystallisation of gelatin which show that super-saturated gelatin solutions do deposit solid gelatin.

It follows that the terms "melting point" and "setting point" have no exact meaning as usually applied to gelatin jellies and solutions. A 1.15 % jelly of ashless gelatin will just dissolve completely if it be maintained at 26.45°. If it be kept at a lower temperature, an amount of gelatin, corresponding to the solubility of gelatin at that temperature will go into solution. But, if the jelly be warmed gradually until melted, it will not melt until the temperature has been raised considerably above 26.45° on account of the slow velocity of solution. Conversely, if a warm 1.15 % solution of gelatin

be cooled, the liquid will not set until the temperature has fallen much below 26.45° , or actually to the room temperature. If the temperature be maintained at a point below 26.45° , the gelatin in excess of the solubility at that temperature will be precipitated. Whether the precipitate will form a jelly, or not, will depend on its amount. Obviously, the observed melting and setting points depend on the rate of heating. Similarly the grain size of the jelly is affected by the temperature at which the bulk of the excess gelatin is precipitated. When a more exact expression for the grain size has been found, it should be possible to deduce the relations between the excess concentration, and the elasticity, vapour pressure and other properties of the resulting gels.

It remains to investigate the nature of the deposited solid. It has been mentioned that, when the particles of gelatin became large enough to see, they are spherical in shape. Von Nägeli observed the beautiful brushes shown by spherites between crossed nicols and described their radiating-needle structure, but he was unable to say whether his micellae were identical with spherites. However, it is now certain that gelatin particles have this conformation. The study of substances which crystallise in this shape, and experimental investigation, show that bodies of high molecular weight, and those that exist in solution in the aggregated state, tend to crystallise in the form of spherites. Indeed, it is obvious that a slow moving, comparatively large particle, is able to orientate itself to fit a growing spherite, composed of radiating needles, more easily than a crystal of another shape. It is reasonable to suppose, therefore, that the granules composing jellies of gelatin and agar are spherites.

Spherites are known in every gradation, from the obviously crystalline form, built up of coarse radiating needles separately visible, through stages showing only a more or less radiating formation and often a banded structure, but giving the well-known shadow-cross in polarised light, to apparently isotropic bodies giving little definite evidence of crystalline structure. Microscopic examination of gelatin spherites proves that they are crystalline and belong to the second class. This completes the evidence that gelation is a crystallisation process.

Granules deposited from dilute solutions of pure ashless gelatin under aseptic conditions may reach 1μ in size. These show perfect shadow crosses when viewed between crossed nicols. It is convenient, however, to grow them larger. This is done exactly in the same way as larger particles of other substances would be grown, *i.e.* by spontaneous evaporation of saturated solutions. In these experiments 0.3 % solutions were made in water containing 0.1 % of mercuric chloride or camphor as antiseptic. The liquid was filtered into chemically clean glass crystallising dishes, which were covered and allowed to stand at room temperature for periods of from one to six months. The liquid becomes opalescent within 24 hours as the precipitation of the gelatin begins. Soon a white, or slightly buff-coloured, powder is seen on the base of the dish. The tint may be due to a trace of pigment, or be the actual colour of the gelatin

particles. Smaller particles always appear white, but it is possible that this may be caused by the greater subdivision. After some time the supernatant liquid is observed to have set to a jelly. This is because evaporation has taken place faster than the gelatin could diffuse through the liquid to the growing spherites. When this happens, further diffusion is practically stopped and the spherites can increase in size only by actual increase in concentration of the liquid in their immediate vicinity, or at the expense of smaller adjacent particles. Consequently the spherites attain nearly their maximum size in a month or two. The precipitate was scraped from the base of the dishes and mounted either in mother liquor, the refractive index of which differs but little from that of the gelatin particles [Walpole, 1913], or glycerol of much higher refractive index, but the spherites appeared to dissolve slowly in the latter medium. The edges of the cover glasses were cemented with Japan gold size. After two months the precipitate consisted entirely of spherites up to 3μ in diameter with a few rather larger grains. They were viewed with a Leitz fluorite $\frac{1}{12}$ inch objective and an eyepiece magnifying twelve times. Between crossed nicols many showed perfect shadow crosses, but, of course, only one or two could be brought into focus at a time. After three months the spherites were up to 3μ or slightly over. There were also a few spherites from each of which a bundle of fibrils had grown like the tail of a comet. Similar appendages are shown occasionally by spherites of silver dichromate. After four months' evaporation, the particles had grown to about 4μ . By six months the larger particles had reached 4.4μ in size. Almost all showed shadow crosses more or less complete. But the crosses were the more perfect the smaller the size of the particles. This gives no reason for supposing that the still smaller particles of jellies are not crystalline. The appearance of the precipitate after two months' growth is shown in the photographs (Figs. 1 and 2, Plate III), for help in taking which the writer is indebted to the kindness of Dr L. C. Martin. The shadow crosses show best when the equators of the particles are exactly in focus, and are altogether different in appearance from those shown by light polarised by reflection at the surfaces of air bubbles. Grains of mastic grown by Perrin's method showed no shadow crosses but only a slight luminescence. By evaporation at 23.5° spherites have since been obtained up to 6μ . This proves that the gelatin spherites are crystalline.

Since agar also separates from solution in the form of granules, it appears probable that the structure of jellies of this substance and of gelatin is that of a pile of shot. Such a fine grained structure is compatible with all the known properties of jellies except the heat of swelling, 5.7 cal. per g. [Wiedemann and Lüdeking, 1885] and the so-called "thermal anomaly" [Bjerkén, 1891]. Re-determination of the former gave a value 33.25 cal., which corresponds to that for starch. Investigation of the thermal anomaly showed that it was unfounded. The pile-of-shot structure offers a simple explanation of the lens-shaped form of gas bubbles in gels investigated

by Hatschek [1914]. Moreover, the crystallisation theory explains another curious phenomenon observed by Hatschek [1920]. It is well known that mechanical strain causes gelatin gels to exhibit optical anisotropy in polarised light. When a rectangular prism of jelly is bent between three stops, the usual coloured appearance is observed in polarised light. After five days in a moist chamber, the mechanical stress disappears, so that the specimen can be removed without straightening itself appreciably. Extraordinarily enough, however, the appearance in polarised light remains unchanged. The explanation may be as follows. Gelatin and agar are remarkable for their stability, although most substances that can be obtained in the gel form, under suitable conditions, develop rapidly microscopic crystals. The permanence of the natural gels is due to the low solubility, the very low rate of diffusion of the gel substances, and to the fact that dissolved material is almost unable to diffuse through the jelly. But, if the crystallisation theory is true, very slow re-crystallisation should be going on. Evidences of slow re-crystallisation in silicic acid gels have been observed previously. They have now been obtained in the case of gelatin.

Optically clear aseptic 5 % gelatin gels were sealed in tubes and kept at room temperature. After three months a distinct opalescence had developed, which grew from month to month until, after ten months, it had become very marked. Microscopic examination after four months showed an almost clear field in which some very tiny round particles could just be seen. With lapse of time the visible particles increased in number and size. In ten months some had reached nearly 1μ in size. It appears, therefore, that there is an extremely slow process of re-crystallisation taking place constantly in gelatin jellies, by which the larger particles are growing continually at the expense of the smaller ones, just as in the case of any ordinary substance in contact with a solvent. Moreover, the marked increase in the modulus of elasticity during the first 24 hours indicates that the separation of gelatin from solution, in the slow process of setting, is not complete even within that time. Since gel substance is being deposited on the larger particles in a gel, whether strained or not, in a strained gel the larger particles will become cemented in their constrained positions. So that, on removal of the stress, the particles will be unable to move, but the internal strain will continue and maintain the optical appearance in polarised light.

In default of evidence to the contrary, therefore, we must conclude that the forces which hold gelatin and agar in solution are the same as those that operate between other solutes and solvents, and that the setting of jellies of the natural emulsoids is merely a process of crystallisation.

The writer desires to express his gratitude to the Government Grant Committee of the Royal Society and to the Chemical Society for grants which have enabled these experiments to be continued.

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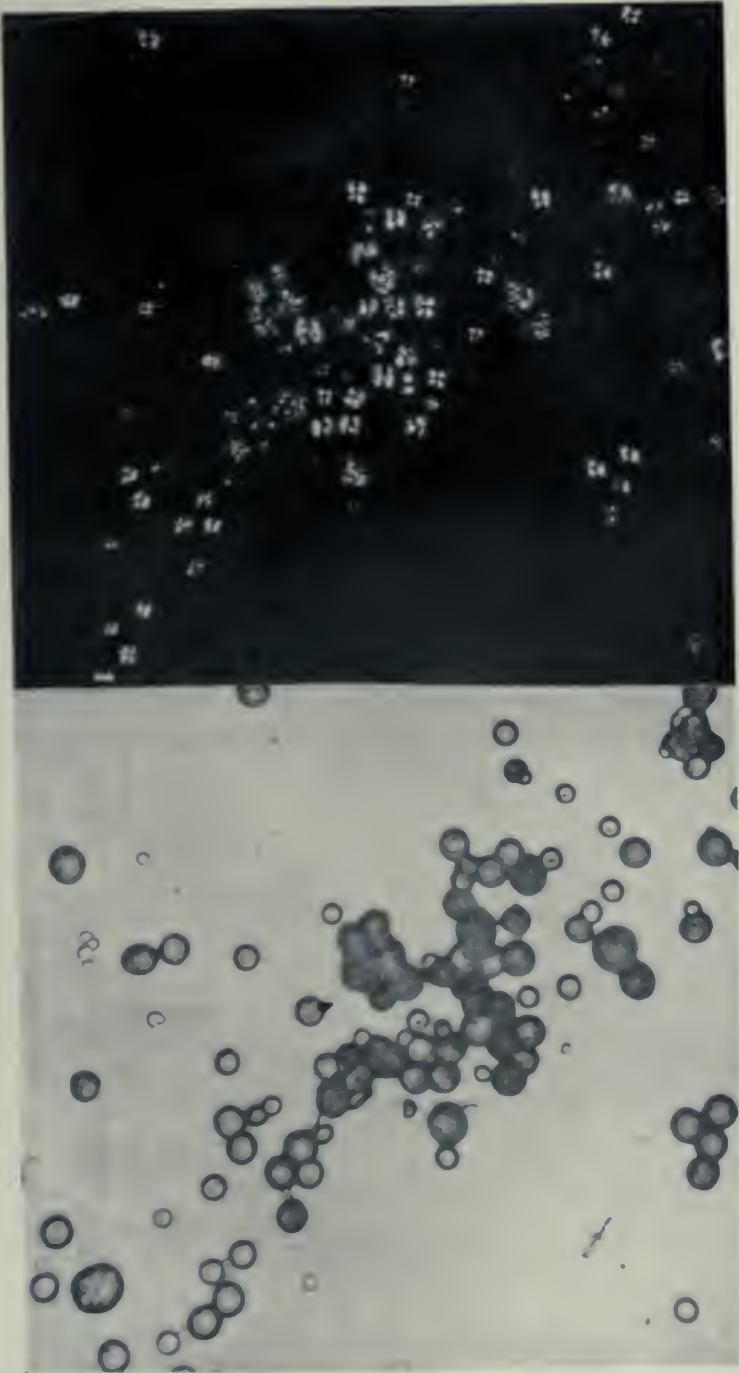


Fig. 1. Gelatin spherulites. $\times 1400$.

Fig. 2. Gelatin spherulites between crossed nicols. $\times 1400$

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XXX. THE BALANCE OF ANIONS AND KATIONS IN THE PLASMA IN NEPHRITIS.

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THIS work was begun with the intention of finding what anions, accumulated in the plasma, were responsible for the occurrence of acidosis, as shown by diminution of plasma $[\text{HCO}_3^-]$, in patients with nephritis. Such an accumulation of anions might be due either to a general impairment of excretion resulting in the retention to a greater or less degree of all the anions which should be excreted in the urine, or to a specific inability to excrete some particular anion resulting in the retention of this anion alone.

In order to decide to what degree any particular anion is responsible for a diminution of plasma $[\text{HCO}_3^-]$ it is necessary to know the concentrations of all the anions and kations in the plasma; for an increase of one anion will not cause an acidosis if accompanied by a corresponding increase of kation or diminution of some other anion. This explains the lack of correspondence found for example by Denis and Minot [1920] between accumulation of phosphate in the plasma and diminution of plasma bicarbonate.

It is therefore necessary to calculate the balance between the anions and kations in the plasma of patients with acidosis, and for purposes of comparison in that of other patients.

In making this balance other points with regard to individual constituents of plasma and the balance between them have arisen and I will consider these besides the original question of the cause of acidosis.

Table I. *Balance of anions and kations in plasma of control cases.*

<i>Anions.</i>					
Average of control cases	$[\text{HCO}_3^-]$	—cc. of CO_2 per 100 cc.	61.0	0.0277 g. equivalents	per litre
	$[\text{Cl}^-]$	—g. NaCl	0.600	0.1026	" "
	$[\text{HPO}_4^{--}]$	—g. P_2O_5	0.0066	0.00165	" "
Denis [1921]	$[\text{SO}_4^{--}]$	—g. S	0.0008	0.0005	" "
	Protein	—and organic acids		0.0105	" "
				Total	0.143
<i>Kations.</i>					
Average of control cases	$[\text{Na}^+]$	—g. Na	per 100 cc.	0.030	0.1305 g. equivalents per litre
Myers and Short [1921]	$[\text{K}^+]$	—g. K	"	0.020	0.005 " "
	$[\text{Ca}^{++}]$	—g. Ca	"	0.010	0.005 " "
	$[\text{Mg}^{++}]$	—g. Mg	"	0.003	0.0025 " "
				Total	0.143

Table II. *Balance of anions and kations in cerebrospinal fluids from patients with no evidence of nephritis. These fluids contained only minute traces of protein.*

<i>Anions.</i>					
Average of these cases	[HCO ₃ ⁻]	—cc. of CO ₂ per 100 cc.	62.0	0.028 g. equivalents per litre	
	[Cl ⁻]	—g. NaCl	0.735	0.126	" "
	[HPO ₄ ⁻⁻]	—g. P ₂ O ₅	0.004	0.001	" "
			Total 0.155		
<i>Kations.</i>					
Average of these cases	[Na ⁺]	—g. Na	per 100 cc.	0.337	0.147 g. equivalents per litre
Myers [1917]	[K ⁺]	—g. K	"	0.022	0.005 " "
Halverson and	[Ca ⁺⁺]	—g. Ca	"	0.005	0.0025 " "
Bergeim [1917]	[Mg ⁺⁺]	—g. Mg	"	0.003	0.0025 " "
			Total 0.157		

The figure given for Mg in cerebrospinal fluid is that for plasma as I can find no estimate of magnesium in cerebrospinal fluid.

BALANCE OF ANIONS AND KATIONS IN NORMAL PLASMA.

The balance of the average values of anions and kations in my control cases is shown in Table I. There is an excess of kations unaccounted for by Cl⁻, HCO₃⁻ and HPO₄⁻⁻ equal to about 0.010 g. equivalents per litre. This is combined with protein and anions of organic acids. Probably organic acids only account for a small fraction of these kations. In cerebrospinal fluid which contains practically no protein, the excess of kations does not occur, as shown in Table II. Also Greenwald and Lewman [1922] have recently published a method by which they estimate the amount of kations in plasma combined with HCO₃⁻ and with protein together. In the normal cases in which the bicarbonate is given, the kations combined with protein are equivalent to 0.0097 and 0.0136 g. equivalents per litre, and are therefore about equivalent to the amount of base in plasma unaccounted for by HCO₃⁻, Cl⁻ and HPO₄⁻⁻.

Kramer and Tisdall [1922] have published a table of average values of the anions and kations in plasma showing the excess of bases unaccounted for as 0.025 g. equivalents per litre. I think it probable that the "average" value taken by them for plasma [Na⁺] is high in proportion to their "averages" for [Cl⁻] and [HCO₃⁻].

I have estimated only the [HCO₃⁻], [Cl⁻], [HPO₄⁻⁻] and [Na⁺]. Calcium may vary between 0.006 and 0.011 %, or between 0.003 and 0.0055 g. equivalents per litre, potassium between 0.010 and 0.020 % or between 0.0025 and 0.005 g. equivalents per litre. Variations of magnesium are negligible as the amount is so small. If, therefore, we add to the [Na⁺] 0.0125 g. equivalents we shall obtain an estimate of the total kations with a maximum error of 0.0045 g. equivalents per litre, not more than 3.5 % of the total kations, and little more than the errors in sodium estimation.

As normally the principal anions, HCO₃⁻, Cl⁻ and HPO₄⁻⁻, are about equivalent to the Na⁺, and therefore the other kations to the normal protein, it is possible to omit these last items in balancing the anions and kations.

Taking them into account merely involves adding two equal amounts to both sides of the equation.

In such a balance an accumulation of Cl^- or HPO_4^{--} in the blood, causing a diminution of plasma bicarbonate, is shown directly; while, if an accumulation of another acid ion, such as SO_4^{--} , occurs, its presence is shown by an excess of Na^+ unaccounted for by the anions estimated. Such an accumulation is illustrated in Table III; the excess of Na^+ is equivalent to the keto-acids within the limits of error of the method.

As I originally intended to consider acidosis only, the patients in this series have been selected mainly from severe cases of nephritis, who might be expected to have some disturbance of their acid base regulation.

Table III.

Excess of Na^+ unaccounted for by HCO_3^- , Cl^- , and HPO_4^{--} in plasma in ketosis.

Case	p_{H}	$[\text{HCO}_3^-]$	$[\text{Cl}^-]$	$[\text{HPO}_4^{--}]$	Sum of three anions	$[\text{Na}^+]$	Excess Na^+	β -Hydroxy- butyric acid and aceto-acetic acid	
Gram equivalents per litre									
60	7.0	0.0055	0.112	0.002	0.1195	0.144	0.0245	0.0215	Diabetic coma; Moderate hyperpnoea. Blood sugar 0.44 %. Died next day.
61	7.4	0.0215	0.099	0.002	0.1225	0.1305	0.008	0.0065	Diabetes; prolonged ketosis without coma. Blood sugar 0.26 %.

Total acetone bodies were estimated by the method of Van Slyke; 20 % was assumed to be present as acetone.

Some cases of heart failure have been included to illustrate the changes that may be found in this condition.

METHODS.

For plasma bicarbonate when determined without p_{H} Van Slyke's [1919] titration method was used. When p_{H} and plasma bicarbonate were determined, methods of Van Slyke [1922] and Cullen [1922].

The blood was obtained through a needle with a short piece of rubber tubing attached; a piece of rubber tubing was fixed tightly round the patient's upper arm. After the needle was inserted the blood was allowed to flow, to allow all congestion from the constriction of the arm to pass off, and the tubing was then connected to the entry tube of a 25 cc. centrifuge tube as shown in Fig. 1. By withdrawing the wide part of this entry tube a short distance from the hole in the stopper, air was allowed to escape as the blood flowed in. The centrifuge tube had previously been filled with alveolar air in order that the CO_2 tension of the oil and any air with which the blood came in contact might approximate as nearly as possible with that of the blood taken. When the oil began to overflow the entry tube was removed, a glass plug inserted,

and the tube inverted three or four times. The effect of the small bubble of air left in the tube would be negligible. The blood was then rapidly centrifuged and the plasma thus obtained was removed with a pipette through the hole in the stopper for p_{H} , bicarbonate, Cl and P_2O_5 determinations. These therefore were determined at the CO_2 tension of the patient's venous blood.

The solutions of known p_{H} used for p_{H} determinations were made according to the formula in Cullen's paper, from pure salts recrystallised. These solutions contained the theoretical amount of phosphorus determined gravimetrically. The p_{H} is given to the nearest 0.05 as it was occasionally difficult to get a more exact match.

Chlorides were estimated by the Volhard method on filtrates after precipitation of protein by trichloroacetic acid. Precipitation with trichloroacetic acid is less complete than with picric and tungstic acids, and in some cases some difficulty was experienced in getting the silver chloride to flocculate satisfactorily, probably owing to proteoses left unprecipitated. In two cases, in one of which the flocculation did not take place readily and in the other the chloride value was very low, the result was checked by an incineration method.

In some cases, when sufficient blood was not available for estimation of chlorides in whole blood, the corpuscles separated from the plasma as above were used for chloride determination. As the separation was done rapidly it is probable that the corpuscles were not completely separated from plasma and that the Cl values found are too high; this would occur especially in the more normal cases in which the corpuscles sink slowly. These figures therefore are upper limiting values only.

Inorganic phosphates. The figures given in brackets were estimated by the original method of Bell and Doisy [1920] before the effect of oxalate was realised. The amount of oxalate used was minimal, and the figures are therefore probably a little too low. Later the modification given by Denis and Meysenbug [1922] and the method of Briggs [1922] were used.

Sodium was estimated by the method of Kramer and Tisdall [1921]. This method gave theoretical results with pure solutions of sodium chloride, duplicates agreed and added sodium chloride was satisfactorily recovered from plasma or serum.

Determinations were mainly made on plasma as more can be obtained from a given amount of blood, and the delay and risk of haemolysis during the separation of serum is avoided by using plasma.

Determinations on plasma and serum from the same blood agreed.

This method is liable to an error of $\pm 3\%$ which is of importance in considering the balance of acids and bases.

Calcium was determined in a few cases by the method of Clark [1921].

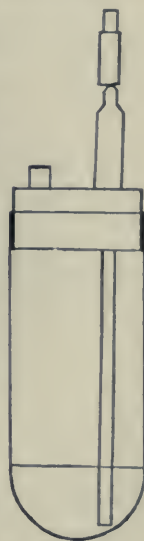


Fig. 1.

Plasma proteins were determined in some of the cases by Dr Villett by the method of Wu [1922, 2]. He has kindly permitted me to use his figures.

Blood urea was estimated by the urease method.

In Table V [HCO_3^-], [Cl^-], [HPO_4^{--}], and [Na^+] are given in g. equivalents per litre; "Sum" represents the sum of the g. equivalents per litre of the anions.

Chlorides in plasma.

The concentration of Cl^- in plasma is affected by changes of the tension of CO_2 which cause a migration of Cl^- between the red corpuscles and plasma, but the changes produced thus are much less than the variations found in these cases. Thus Doisy, Eaton and Chouke [1922] found the plasma [Cl^-] fall from 0.65 to 0.61 % NaCl (0.111 to 0.104 g. equivalents per litre), in human blood with an increase of CO_2 tension from 23.3 to 72.5 mm. These values for CO_2 tension are extreme; such deviations from normal CO_2 tensions do not occur in normal persons, and were only found in the extreme cases in this series. In normal persons, also, variations of plasma [Cl^-] occur much greater than those caused by variations of CO_2 tension. Thus in the series given by Wu [1922, 1] the values ranged from 0.56 to 0.67 % NaCl—0.096 to 0.1145 g. equivalents per litre. His figures for the Cl in the corpuscles also show that the variations in plasma were not due to migration of Cl^- in and out of the corpuscles, as high concentrations in plasma were usually associated with high values in corpuscles and *vice versa*.

The variations found by Wu are greater than those usually found, *e.g.* by Norgaard and Gram [1921], although Wu and Norgaard and Gram are in fair agreement as to the average value, about 0.62 % or 0.104 g. equivalents per litre.

The plasma Cl in this series was on the whole low. Out of 98 determinations 27 were under 0.096 g. equivalents per litre.

In another series of 100 patients taken without selection of the more severe cases the plasma [Cl^-] was under 0.096 g. equivalents per litre in 7.

All patients with plasma [Cl^-] under 0.092 g. equivalents per litre were severely ill, and those with values under 0.0855 still more so. In cases such as (31), (32), (38) and (39) which steadily became worse the plasma [Cl^-] steadily fell; this did not always occur however, as for example in case (33). Cases (6) and (37) had very low figures when at their worst with an increase on improvement.

There is no relation between the type of nephritis and the plasma [Cl^-]. Oedema occurred both with normal and low values.

There is no evidence that the variations were due to variations of CO_2 tension or reaction of the plasma or to migration of Cl^- between plasma and corpuscles¹. Thus case (47) with an alkaline plasma had a plasma [Cl^-]

¹ It is not suggested that migration of Cl^- between corpuscles and plasma did not occur, but that it was overshadowed by other changes.

0.093 g. equivalents per litre and the low plasma $[\text{Cl}^-]$ of cases (31), (32) and (39) was associated with low values for Cl in the corpuscles.

Phosphates in plasma.

The plasma $[\text{HPO}_4^{--}]$ varies in the same sense as the blood urea but not proportionately; which is to be expected from the large and variable excretion of phosphate which takes place in the faeces also. Two cases, (39) and (40), especially have high blood urea with no increase of plasma $[\text{HPO}_4^{--}]$. In these cases Cl appears to have been stored in the tissues and it is possible that a similar storage of HPO_4^{--} occurred; but one cannot exclude the possibility that the excretion of HPO_4^{--} may have been performed completely by the intestine, so that there was actually no retention. It is remarkable that in these two cases the kidneys were entirely out of action.

Sodium in plasma.

Two values for normal sodium concentration in plasma are found in the literature. Those of Kramer [1920] 0.28 to 0.31 %, 0.122 to 0.135 g. equivalents per litre, and those of Kramer and Tisdall [1921], 0.326 to 0.350 %, with average of 0.33%, 0.142 to 0.152, with average 0.144, g. equivalents per litre. My figures for the mild cases of nephritis and cases without evidence of kidney damage agree better with the first.

The values found in this series were on the whole low, but reductions of $[\text{Na}^+]$ as great as those of $[\text{Cl}^-]$ did not occur. Except in case (37) (10. viii. 22) the reduction of $[\text{Na}^+]$ was not more than equivalent to that of $[\text{Cl}^-]$ in the same patient.

It is remarkable that, as has long been known for Cl, as is shown by Myers and Short [1921] for K, various workers for Ca and by these results for Na, no accumulation of these constituents occurs in the plasma in nephritis, even with extreme degrees of renal inefficiency, which are accompanied by great increase of plasma urea and HPO_4^{--} . This points to methods of regulation of the amounts of the former substances in the plasma other than excretion through the kidneys; which methods may be retention of water, and deposition in the tissues.

In case (39) the water retention must have been inadequate to keep down the Cl concentration; the NaCl retained during 15 days on ordinary diet with no restriction of Cl intake must have been at least 45 g., which would require 11 litres of water to dilute it to the concentration of the plasma at death; an amount which could not have been retained without gross oedema. The second process therefore, deposition in the tissues, must have played a large part. His Na intake was increased by large doses of sodium bicarbonate, the absorption of which is proved by increase of the plasma bicarbonate to normal, and its subsequent maintenance at normal in spite of increasing amounts of undetermined acids in the plasma; but the plasma $[\text{Na}^+]$ did not increase,

instead towards the end it fell. Accompanying this there was a steady fall of $[\text{Cl}^-]$. This suggests that Na also was deposited in the tissues so that, instead of the plasma bicarbonate being increased by the absolute increase of $[\text{Na}^+]$ in the plasma, it was increased by removal of Cl^- into the tissues along with the extra base supplied, leaving a relative excess of Na^+ in the plasma.

A similar effect of treatment with bicarbonate is seen in other cases, (13), (26), (30) and (35), although in these we have not such satisfactory evidence that the result is not brought about by water retention; in case (35) especially the output of water during two days of bicarbonate administration fell below the intake by 2 litres, although after the administration was stopped output was equal to intake.

The low values of plasma $[\text{Cl}^-]$, and to a less degree of plasma $[\text{Na}^+]$, point to some disturbance of the mechanisms that regulate these constituents. It is probable that in most of the cases water retention played a large part; such water retention would explain the low values of cell volume and total proteins¹. It is probable, however, that tissue deposition, as in (39), occurs also in other cases; in case (31) for example, in which output equalled intake in spite of profuse sweating until the last three days, water retention alone does not seem adequate to explain the fall in plasma $[\text{Cl}^-]$ which occurred in spite of retention of about 1 g. of NaCl a day.

There is further evidence of such a deposition of chloride in the tissues in the high Cl content of the heart muscle in cases (39) and (33)—0.24 and 0.31 % of sodium chloride as compared with 0.16 and 0.19 in the hearts of two healthy men who were killed suddenly in accidents. Such deposition in the tissues was shown by Wahlgren [1909] to occur in dogs after large doses of sodium chloride; he found however that the percentage increase in muscle was less than in other tissues, so that had some other organ been chosen for analysis in these two cases the increase found might have been greater. In cases (26), (32), (33) there is a striking increase in the $[\text{Cl}^-]$ and $[\text{Na}^+]$ in the cerebrospinal fluid in comparison with that in the plasma.

In cases (3), (6), (31), (32) and (38) the excretion of Cl in the urine was continued although the plasma Cl had fallen well below the recognised threshold.

It is reasonable to suppose that these changes are all due to some alteration of the mechanism which regulates the distribution of sodium chloride between plasma and other body fluids. It is possible that this is the result of changes in the plasma proteins.

The low figures found for $[\text{Cl}^-]$ and $[\text{Na}^+]$ are of great importance in the balance of acids and bases. Such changes make it impossible to calculate what effect accumulation of some acids such as phosphoric will have on the plasma bicarbonate unless these constituents also are estimated.

¹ The change in plasma proteins however is more than a mere reduction of total protein; there is a change in the proportions of the protein fraction and actual increase of fibrinogen.

BALANCE OF ACIDS AND BASES.

In the cases of hyperpiesia the relation between the sum of the anions estimated and the sodium is the same as in the control cases. In the mild cases of nephritis (2), (4), (6), (14) and (15) there is slightly more sodium in relation to anions but not enough to justify any conclusion. Also in several of the more severe cases, (26) (except on 27. iv. 22), (30), (34) and (38) (on 28. vii. 22) the relation differs little from that in the controls.

Table IV.

	Average values in g. equivalents per litre				
	[HCO ₃ ⁻]	[Cl ⁻]	[HPO ₄ ⁻]	Sum	[Na ⁺]
Control	0.0274	0.1026	0.0016	0.1316	0.1305
Hyperpiesia	0.032	0.099	0.002	0.133	0.131
Mild cases	0.029	0.103	0.002	0.134	0.1365

But in 18 cases (1, 9, 12, 13, 18, 23, 25, 26, 28, 29, 31, 32, 33, 35, 39, 40, 41 and 52), the Na exceeded the sum of these three anions by more than 0.0085 g. equivalents per litre, the maximum error to which this method of estimating the total kations is liable. Of these, 16 were frankly severe cases with blood urea raised. Of the remaining two, one case, (52), had severe heart failure, and one case, (18), was an old man with an enlarged prostate and considerable impairment of renal efficiency as tested by phenolsulphone-phthalein excretion and urea concentrating power.

A steady increase of this excess of kations is seen in cases (31), (32) and (39) as the blood urea rose, while in cases (6) and (1) the excess of kations fell as the patient improved and the blood urea fell.

This excess of kations may be combined (1) with protein or (2) with undetermined acid ions.

The changes in plasma proteins that occur in nephritis are reduction in total protein and albumin, increase of fibrin and a relative and sometimes absolute increase of globulin. These changes are seen in cases (7), (9), (11), (31), (32) and (33). It is difficult to see how such changes can result in an increase of kations combined with protein unless we suppose the fibrin has a much greater power of combining with kations than have albumin and globulin. In case (32) the excess of kations was much greater when the total protein was 6.04 % than when it was 7.89 %; in case (33) with high fibrin there was no excess of Na⁺.

In the cerebrospinal fluids of cases (26), (32) and (33) there is no excess of kations, as in the plasma taken before and after, but the difference between the sum of the three anions and the Na⁺ is the same as in normal cerebrospinal fluids. This might be due to the absence of protein, but can be just as well explained by supposing that there is a much lower concentration of the unknown anions in cerebrospinal fluid than in plasma, just as is the case with phosphoric acid, and as found by Berglund [1922] with amino-acids, creatine and uric acid.

It is therefore probable that the excess of kations is not combined with protein but with some anions which have not been determined.

Undetermined acid in the form of keto-acids probably accounts for the excess in case (40) and possibly in the form of lactic acid in cases (26) and (52).

In the other cases it is probable that no one anion is responsible, but a mixture of anions which should be excreted in the urine—among these SO_4^{--} which has been found by Denis [1921] in nephritic blood in sufficient amount to produce this effect.

In the ascitic fluid of case (39), post mortem there were 12 mgm. S as sulphate per 100 cc., or 0.0075 g. equivalents per litre. Such a concentration would account for part of the excess of kations of 0.021 g. equivalents per litre found in the plasma.

However this sulphate may have been produced by bacterial changes which had taken place in the fluid.

It is hoped to evolve a method of sulphate estimation applicable to the small quantities of plasma available.

Plasma bicarbonate and reaction.

Owing to the migration of Cl^- between red corpuscles and plasma with variations of the CO_2 tension, it is necessary to have some method of distinguishing low plasma bicarbonate due to accumulation of acid in the blood from low plasma bicarbonate due to the exposure of an otherwise normal blood to abnormally low CO_2 tensions with consequent migration of Cl^- into the plasma. Charts I and II serve this purpose. Abscissae represent venous CO_2 tension, ordinates $[\text{HCO}_3^-]$ in g. equivalents per litre. Since the hydrogen ion concentration of plasma is proportional to the ratio of the CO_2 tension to the plasma bicarbonate, the points representing plasmata with the same hydrogen ion concentration and therefore the same p_{H} must all lie on a straight line passing through the origin. Such lines for p_{H} 7.1 to 7.6 are shown; they were calculated by the formula given by Austin *et al.* [1922].

The point representing a plasma can be determined by its $[\text{HCO}_3^-]$, giving the ordinate, and p_{H} , giving the p_{H} line on which it lies, or CO_2 tension, giving the abscissa.

Points for plasmata separated from normal bloods at varying CO_2 tensions may lie within the shaded area. The two curves shown are taken from the paper of Doisy, Eaton and Chouke [1922] and the limits of the normal area are calculated from the limits of the normal dissociation curves given by Peters, Barr and Rule [1921]. If a low $[\text{HCO}_3^-]$ is due to the separation of the plasma from otherwise normal blood at low CO_2 tension, the point for the plasma will still fall inside the normal area, while if it is due to accumulation of anions in the blood (acidosis) the points will fall below the normal area. If there is an excess of kations in the blood (alkalosis) the points will fall above the normal area.

The normal p_{H} lies between 7.3 and 7.4 the average being nearer 7.4. Normal cases should therefore fall between these two lines.

The plasmata of control cases all fall within the normal area, and with the exception of case (56) have normal p_{H} . Case (56) is an example of the effects of hyperpnoea reducing the venous CO_2 tension and causing a high p_{H} and comparatively low plasma $[\text{HCO}_3^-]$. A similar condition has been produced outside the body in case (59) by not taking precautions to avoid loss

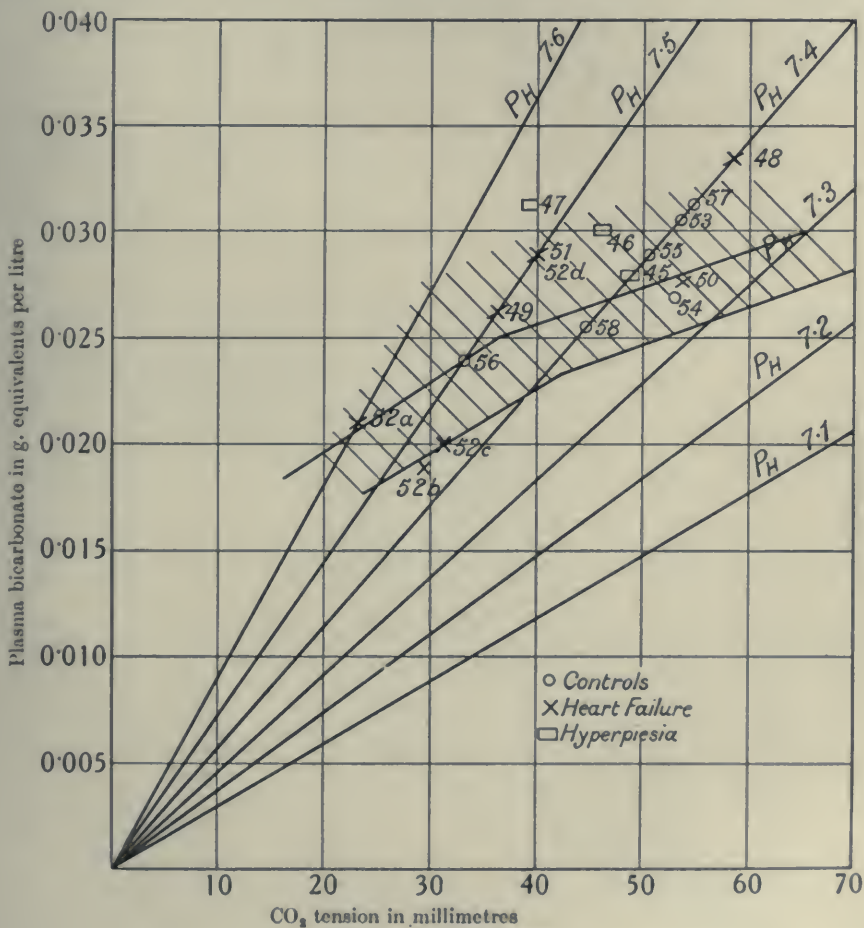


Chart I. Control cases.

of CO_2 . In the balance of acids and bases in these two cases we find evidence of the migration of Cl^- into the plasma in the increase of $[\text{Cl}^-]$ above the average without corresponding increase of $[\text{Na}^+]$. As closely as can be expected with the errors to which the method is liable the fall in $[\text{HCO}_3^-]$ is accounted for by this relative increase of $[\text{Cl}^-]$.

In the cases of heart failure two (48) and (52) (b) and (c) fell at the upper and lower limits of the normal area and three (49), (51) and (52) have high p_{H} .

spite of increased hyperpnoea, while there was an accumulation of undetermined anions resulting in reduction of the $[\text{HCO}_3^-]$; in consequence of these two changes the plasma p_{H} fell. With great clinical improvement on 14. xi. 22 her $[\text{HCO}_3^-]$ rose above the value on 4. xi. 22 but her CO_2 tension remained still somewhat low although the hyperpnoea was hardly noticeable.

In cases (49) and (52) the migration of Cl^- is not detectable, being obscured by other changes.

The plasmata of two cases, (45) and (46); of the three cases of hyperpiesia in which the p_{H} was determined, fell in the normal area, and of one (47), who had been treated with large doses of sodium bicarbonate, above this area.

Cases (46) and (47) with high p_{H} had disturbances of the central nervous system which may have affected their respiratory centres. Case (47) was also in considerable distress from violent headache, which may also have caused hyperpnoea. In these two cases the excess of plasma $[\text{Cl}^-]$ and low bicarbonate are not seen; in case (47) the absence of high plasma $[\text{Cl}^-]$ is explained by the low $[\text{Cl}^-]$ in the whole blood, so that even with a lower proportion of this Cl^- than usual in the corpuscles that in the plasma would not be high.

In all the mild cases of nephritis (Chart II) the plasma $[\text{HCO}_3^-]$ fell within the normal area. The average $[\text{HCO}_3^-]$ for the five cases (2, 4, 5, 14 and 15) was 0.0287 g. equivalents per litre which agrees closely with that for the controls. In one case (15) (pyelitis of pregnancy) the plasma p_{H} was high. It is not improbable that a slight degree of hyperpnoea and consequent high plasma p_{H} occurs in pregnancy apart from any pathological condition. In this case as in case (47) the $[\text{Cl}^-]$ in the plasma and blood was low.

Of the severe cases also a large number fell in the normal area; among these are cases (26, *b*) (2. v. 22), (33), (34), (39 *d*) (29. vi. 22) with high plasma $[\text{HPO}_4^{--}]$ or undetermined anions showing that in these cases kation had been retained in sufficient amount to neutralise these anions. Cases (3), (22 *a* and *b*) (5. vii. 22 and 13. vii. 22) (38 *b* and *c*) (3. vii. 22 and 28. vii. 22) and (35 *b*) fall above the normal area; cases (9), (13), (32), (35 *a*) (16. xi. 22), (39 *a*) (21. vi. 22) and (40) fall very low, and cases (31), (20 *a*) (23. vi. 22), (26 *a* and *d*) (27. iv. 22 and 17. vii. 22), (36), (37 *b*) (10. viii. 22) and (23 *a*) (28. vi. 22) below the normal area.

In the majority the plasma p_{H} was normal, in (11 *a*) (3. viii. 22), (22 *a*) (5. vii. 22) and (26 *b*) (2. v. 22) slightly raised, and in (3), (35 *b*) (20. xi. 22), and (33 *b*) (26. x. 22) very high. Of these, cases (22 *a*) and (35 *b*) who had received large doses of sodium bicarbonate, had a high plasma $[\text{HCO}_3^-]$ not fully compensated by increased CO_2 tension. Case (3) who had also received large doses of sodium bicarbonate had a temperature of 101° accompanied by definite hyperpnoea.

Case (33 *b*) (26. x. 22) had heart failure with extreme dyspnoea and is therefore comparable to the case (52 *a*). On treatment with morphia, which presumably deadened his respiratory centre, the dyspnoea was much reduced and his plasma p_{H} returned to normal. Other cases of heart failure with

nephritis, cases (21), (23) and (38), do not show a high p_{H} so that the increased respiratory effort in these cases was ineffective, or had less effect than other changes.

The cases which fell very low in the charts had abnormally low values for plasma p_{H} —7.1 and 7.2—that is to say their respiratory mechanism failed to reduce their CO_2 tension in proportion to their low plasma $[\text{HCO}_3^-]$.

Returning to the cases which fell outside the normal area. Those, (3), (22 *a* and *b*) (5. vii. 22 and 13. vii. 22) (35 *b*) (20. xi. 22) and (38 *b* and *c*) (3. vii. 22 and 28. vii. 22), falling above the normal area had all received doses of sodium bicarbonate which resulted in high plasma $[\text{HCO}_3^-]$ levels. In all the plasma $[\text{Cl}^-]$ is low and the $[\text{Na}^+]$ not increased, that is the high plasma bicarbonate has been produced not by an actual but by a relative increase of kations in the plasma and a reduction of anions in the shape of Cl^- . Case (38 *b* and *c*) had heart failure and oedema of the lungs with very high CO_2 tension compensating his high plasma $[\text{HCO}_3^-]$.

The cases falling below the normal limit are the true cases of acidosis. With them may be included cases (12) and (24); in their case the plasma p_{H} has not been estimated but there is no reason to suppose that they were cases with low plasma $[\text{HCO}_3^-]$ consequent upon reduction of the venous CO_2 tension by hyperpnoea. There were, therefore, in this series of 41 cases with definite kidney damage, 14 with acidosis. The large proportion in this series is due to the severity of the cases chosen. In another series of 100 cases which included a larger proportion of mild cases there were 22 with plasma bicarbonate below 55, and these cases may not all have been true cases of acidosis as some of the less low plasma bicarbonates may have been secondary to reduced CO_2 tension only. The severity of the condition is shown by the fact that of these twenty-two, out of five who had plasma bicarbonate between 35 and 44, four died within two weeks, and out of the five who had plasma bicarbonate below 35 all died within a few days. This heavy mortality also occurred among the cases considered in this paper; of the six cases with severe acidosis four died within fourteen days, and one within a month.

By comparison of the anions and kations in the plasmata of these cases with those of the control cases, we find that the diminution of plasma $[\text{HCO}_3^-]$ is due in the majority of cases to excess of HPO_4^{--} and anions of undetermined acids. Only in two cases (24) (on both occasions) and (23) (on one occasion) does excess of Cl^- alone account for this diminution, and in four an absolute or relative excess of Cl^- together with an excess of HPO_4^{--} or undetermined anions. In four, (13), (32 *a*), (35) and (40), out of the six cases in which the $[\text{HCO}_3^-]$ was extremely low the reduction was wholly due to HPO_4^{--} and undetermined anions; of the other two, the reduction was mainly due to undetermined anions in case (9), while no conclusion is possible in case (39) as the analysis was not completed.

In five cases, (12), (13), (31), (32) and (40) the effect of the increase of these anions was modified by a reduction of $[\text{Cl}^-]$ both absolute and relative to

[Na⁺]. In view of what has been said about the distribution of Cl⁻ it is not justifiable to conclude that there has been no retention of Cl⁻ in these cases, but in view of the high values for HPO₄⁻⁻ and undetermined anions it can be said that there has been no specific retention of Cl⁻ in cases other than (24), and (23) on 28. vi. 22. Even in this last case on the second occasion, 14. vii. 22, there was an increase of undetermined anions and HPO₄⁻⁻ in the plasma and none of Cl⁻.

In all the cases the excess of HPO₄⁻⁻ was less than the excess of undetermined anions or the excess of Cl⁻ relative to Na⁺, so that the reduction of [HCO₃⁻] is much greater than the excess of HPO₄⁻⁻ would account for.

The CO₂ tension of the cerebrospinal fluid must be about the same as that of venous blood, so that in order to maintain the p_H of the fluid the same as that of plasma the [HCO₃⁻] must be the same also. The average values found for the [HCO₃⁻] in cerebrospinal fluid and plasma were about the same 0.0276 and 0.0280 g. equivalents per litre, and in the cases (25), (27), (32) and (33) in which the cerebrospinal fluid was examined at about the same time as the plasma the two agree closely. But in case (32) the low [HCO₃⁻] in the cerebrospinal fluid is accounted for by excess of Cl⁻ instead of high HPO₄⁻⁻ and undetermined anions, as in the plasma. Presumably, as in the process of secretion of cerebrospinal fluid the [HPO₄⁻⁻] and undetermined anions are kept low, the bicarbonate has to be reduced by a relative excess of Cl⁻.

Of the patients with acidosis the only ones with dyspnoea were cases (23), with heart failure, and (37 *b*) (10. viii. 22). On the other hand, dyspnoea occurred in cases such as (22 *a*) (5. vii. 22) with high [HCO₃⁻] and p_H . This dyspnoea therefore, as in the case of heart failure (52), was independent of the reaction and [HCO₃⁻] of the plasma. Dyspnoea appears more as a manifestation of disturbance of the circulatory and respiratory apparatus than of acidosis. Moreover the patients with severe acidosis had little hyperpnoea. It was not noticeable in case (9) when she was sitting in the ward after coming up to hospital and it was not striking in the extreme cases (35 *a*) (16. xi. 22) and (13 *a*) (20. vii. 22). None of the cases had the extraordinary air hunger often seen in diabetic acidosis.

Although acidosis only occurred in severe cases it seemed, like urea retention, to cause little disturbance in itself, even when the p_H of the plasma was reduced. Case (13 *a*) (20. vii. 22) was quite clear-headed and case (35 *a*) (16. xi. 22) was only a little drowsy. Of the more severe cases of acidosis only case (40), who had a ketosis, was comatose; while case (9) was able to go about her work and case (24) (27. ii. 22) was able to get about.

Acidosis was not always present in uraemia, for example it was not present in cases (6), (11) and (29).

Table V.

Case	Plasma							Corpuscles		Blood		Cell volume	
	pH	[HCO ₃ ⁻]	[Cl ⁻]	[HPO ₄ ⁻]	Sum	[Na ⁺]	[Cl ⁻]	[Cl ⁻]	Blood urea				
1	31. v. 22	—	0.030	0.088	(0.0035)	0.121	0.135	—	—	120	40	Acute nephritis. Much improved.	
2	8. vii. 22	—	0.023	0.096	0.0025	0.121	0.126	—	—	54	40	Acute nephritis. Oedema.	
	21. xii. 21	—	0.030	0.102	(0.002)	0.134	—	—	—	25	—	Much improved.	
3	22. viii. 22	7.55	0.0285	0.1105	(0.002)	0.140	0.139	—	—	24	33	Exacerbation of subacute nephritis. Slight oedema. Sodium bicarbonate given by mouth.	
4	6. ii. 22	—	0.032	0.090	0.003	0.125	0.133	—	0.0735	141	—	Subacute nephritis; no oedema.	
	11. i. 22	—	0.0295	0.109	(0.0025)	0.141	0.146	—	—	75	40	"	
5	6. ii. 22	—	0.026	0.102	(0.002)	0.130	0.130	—	—	38	—	"	
6	3. vii. 22	7.4	0.030	0.106	(0.002)	0.138	0.144	—	—	19.5	50	Little change.	
	13. vii. 22	7.3	0.027	0.0785	0.003	0.109	0.117	—	—	120	40	Infective endocarditis; uraemia; NaCl in urine 0.05 %.	
7	26. vii. 22	7.4	0.030	0.102	0.002	0.134	0.1305	—	—	54.5	33	Free diuresis 5. vii. 22 to 7. vii. 22, great clinical improvement at same time.	
	13. ix. 22	7.4	0.0265	0.1005	0.002	0.129	0.1305	—	—	31	33	Still better.	
8	28. ii. 22	—	0.0275	0.1105	0.0025	0.140	0.142	—	—	43	25	Subacute nephritis; oedema. Plasma fibrin 0.31 %, globulin 1.53 %, albumin 2.15 %.	
	25. i. 22	—	0.033	0.104	(0.002)	0.139	0.139	—	—	18	33	Subacute nephritis; oedema. Fits—probably hysterical.	
9	25. i. 22	—	0.024	0.112	(0.0025)	0.138	0.142	—	—	96	50	Congenital cystic kidneys. Able to live moderately active life.	
10	8. iii. 22	—	0.025	0.114	0.002	0.141	0.142	—	—	102	40	—	
	26. x. 22	7.2	0.015	0.117	0.002	0.132	0.142	—	0.1006	141	33	No dyspnoea, nor hyperpnoea. Plasma fibrin 0.31 %, globulin 0.77 %, albumin 3.38 %.	
11	25. i. 22	—	0.027	0.107	(0.002)	0.136	0.139	—	—	47	40	Subacute nephritis; no oedema. Serum Ca 0.010 %.	
	3. viii. 22	7.45	0.025	0.099	0.002	0.126	0.126	—	—	36	—	Pyelonephritis; one kidney removed 7 years ago. Occasional fits.	
12	10. x. 22	7.40	0.0265	0.101	0.003	0.130	0.133	—	0.0795	41	—	Plasma fibrin 0.31 %, globulin 1.12 %, albumin 2.16 %.	
	29. xii. 21	—	0.028	0.109	(0.003)	0.140	0.144	—	—	109	33	Pyonephrosis.	
13	12. i. 22	—	0.022	0.078	(0.006)	0.106	0.128	—	—	109	25	Much worse. Died 21. i. 22.	
	20. vii. 22	7.1	0.007	0.092	0.008	0.107	0.126	0.0565	—	320	—	Pyonephrosis; slight oedema. Fully conscious, no fits. Given 30 g. sodium bicarbonate in next 48 hours.	
14	22. vii. 22	7.2	0.013	0.088	0.009	0.110	0.126	—	—	320	16	Ca in serum 0.006 %. Fits started 8 hours later. Died next day.	
15	2. iii. 22	—	0.034	0.102	—	0.136	0.135	—	—	22.5	—	Pyonephrosis.	
	24. viii. 22	7.5	0.029	0.096	0.002	—	0.126	—	0.0742	25	50	Mild pyelitis. Treated with sodium bicarbonate.	
16	—	—	0.035	0.097	(0.002)	0.134	0.142	—	—	25	—	Chronic interstitial nephritis.	
17	9. i. 22	—	0.030	0.102	(0.002)	0.134	0.128	—	—	41.5	—	Enlarged prostate. Fairly good condition.	
	—	—	0.0335	0.096	(0.002)	0.131	0.130	—	—	37.5	—	—	

18	10. xii. 21	—	0.02635	0.1105	—	0.137	0.148	—	47	—	Enlarged prostate. Not considered fit for operation.
19	—, viii. 22	7.4	0.0295	0.049	0.002	0.1305	0.124	—	26	40	Chronic interstitial nephritis.
20	23. vi. 22	7.3	0.019	0.107	0.002	0.128	0.135	—	43	50	Chronic interstitial nephritis. Just recovering from fit, cyanosed.
21	28. vii. 22	7.4	0.0285	0.104	0.003	0.135	0.130	—	40	40	Much better.
22	5. vii. 22	7.4	0.0245	0.109	0.002	0.135	0.133	—	63	30	Chronic interstitial nephritis. Heart failure. Dyspnoea.
23	13. vii. 22	7.4	0.0365	0.092	0.0025	—	—	—	58	40	Chronic interstitial nephritis. Moderate dyspnoea. Given 60 grains each of sodium bicarbonate and potassium citrate 3 times a day from 24. vi. 22.
24	28. vi. 22	7.35	0.022	0.113	0.002	0.137	0.135	—	83	40	Chronic interstitial nephritis. Heart failure, slight oedema. Dyspnoea.
25	14. vii. 22	7.4	0.023	0.097	0.003	0.123	0.133	0.048	100	33	No oedema.
26	16. i. 22	—	0.018	0.1145	0.002	0.1345	0.135	—	126	40	Chronic interstitial nephritis. Ascites; no oedema.
27	21. xii. 21	—	0.015	0.1215	0.002	0.1385	0.139	—	—	—	Globulin 0.3 % albumin 1.2 %.
28	27. ii. 22	—	0.014	0.1215	0.003	0.1385	0.139	—	120	40	Able to get about; no dyspnoea, slight hyperpnoea.
29	21. xii. 21	—	0.032	0.090	0.002	0.124	0.135	—	75	—	Chronic interstitial nephritis. Coma. No dyspnoea.
30	27. iv. 22	7.4	0.021	0.108	0.002	0.131	0.144	—	106	50	Chronic interstitial nephritis. Comatose. Serum Ca 0.010 %.
31	27. iv. 22	—	0.022	0.135	0.001	0.158	0.146	—	—	—	(Given sodium bicarbonate 30 grains every two hours from 27. iv. 22 to 2. v. 22.)
32	2. v. 22	7.45	0.030	0.100	0.002	0.132	—	—	75	40	Much better.
33	26. v. 22	—	0.022	0.100	—	—	—	—	92	50	Blood equilibrated with 6 % CO ₂ before separation of plasma.
34	23. vi. 22	7.3	0.025	0.101	0.0025	0.128	0.126	—	105	33	—
35	17. vii. 22	7.4	0.020	0.106	0.005	0.131	0.128	—	105	33	Coma and convulsions again; blood taken just after a convulsion.
36	9. x. 22	7.4	0.030	0.102	0.0015	0.133	0.130	0.060	50	—	Chronic interstitial nephritis.
37	27. iv. 22	—	0.032	0.124	0.001	0.157	0.142	—	—	—	—
38	27. iv. 22	—	0.30	0.093	0.0025	0.125	0.148	—	120	25	Chronic interstitial nephritis. Drowsy; no dyspnoea.
39	31. vii. 21	—	0.031	0.082	(0.0075)	0.1205	0.135	—	152	—	Probably pyonephrosis. Unconscious; muscular twitching. Died 2. viii. 2.
40	16. vi. 21	—	0.0275	0.1105	(0.003)	0.140	—	—	131	—	Chronic interstitial nephritis.
41	12. vii. 21	—	0.042	0.092	(0.004)	0.138	0.141	—	150	—	Given large doses of sodium bicarbonate from 7. vii. 21 to 14. vii. 21 then smaller doses.
42	21. vii. 21	—	0.039	0.092	(0.0045)	0.135	—	—	138	—	—
43	2. vii. 21	—	0.0305	0.099	(0.004)	0.134	—	—	155	—	—
44	1. ix. 21	—	0.035	0.096	(0.0045)	0.135	0.139	—	172	—	Died 26. ix. 21 of bronchopneumonia.
45	21. viii. 22	7.3	0.0185	0.093	0.008	0.119	0.124	0.058	300	33	Chronic interstitial nephritis. No oedema, nor dyspnoea.

Table V continued.

Case		Plasma					Corpuscles		Blood	
		pH	[HCO ₃ ⁻]	[Cl ⁻]	[HPO ₄ ⁻]	Sum	[Na ⁺]	[Cl ⁻]	[Cl ⁻]	Cell volume
31	26. viii. 22	7.35	0.020	0.090	0.008	0.118	0.124	—	0.0735	30
	30. viii. 22	7.35	0.0195	0.083	0.0080	0.110	0.124	—	0.0725	600
	4. ix. 22	7.4	0.021	0.085	0.007	0.113	0.137	—	0.0735	600
	11. ix. 22	7.4	0.0165	0.077	0.011	0.104	0.128	—	0.0666	640
32	6. ix. 22	7.2	0.0125	0.096	0.011	0.119	0.133	0.0633	—	436
Spinal fluid }	7. ix. 22	—	0.017	0.135	0.002	0.154	0.144	—	—	456
	8. ix. 22	7.4	0.016	0.087	0.01	0.115	0.131	—	0.077	564
	13. ix. 22	7.35	0.0185	0.074	0.018	0.110	0.142	0.050	—	600
33	8. vii. 22	7.4	0.027	0.096	0.003	0.126	0.130	—	—	57
	26. x. 22	7.6	0.0215	0.106	0.004	0.131	0.135	—	0.0923	171
	31. x. 22	7.4	0.0245	0.104	0.0055	0.134	0.144	—	0.0915	240
Spinal fluid }	6. xi. 22	—	0.0245	0.1455	0.002	0.172	0.165	—	—	—
	34 31. x. 22	7.4	0.028	0.096	0.004	0.128	0.133	—	0.0735	171
35	16. xi. 22	7.2	0.006	0.1025	0.005	0.1135	0.135	—	0.098	343
	20. xi. 22	7.55	0.0335	0.084	0.005	0.1225	0.139	—	0.0785	400
36	1. v. 22	7.4	0.020	0.116	(0.003)	0.139	0.146	—	—	68
37	6. vii. 22	7.4	0.0275	0.106	0.004	0.137	0.137	—	—	70
	21. vii. 22	—	0.015	0.104	0.0035	0.122	0.119	—	—	71
	10. viii. 22	7.4	0.0195	0.094	0.0055	0.119	0.119	—	0.083	111

Given 20 grains sodium bicarbonate and 10 grains potassium citrate 3 times a day throughout; sweated much in hot packs.

Plasma fibrin 0.53 %, globulin 2.41 %, albumin 3.62 %.

Appears better. Plasma fibrin 0.59 %, globulin 2.18 %, albumin 3.43 %.

Worse, comatose. Plasma fibrin 0.61 %, globulin 1.37 %, albumin 2.72 %. Slight oedema of ankles at end. Died 13. ix. 22.

Chronic interstitial nephritis.

Given 30 grains acid sodium phosphate every 4 hours from 2. ix. 22 to 7. ix. 22, then large doses of sodium bicarbonate.

Plasma fibrin 0.36 %, globulin 4.02 %, albumin 3.5 %. Possible loss of CO₂ in taking this blood. Urine NaCl 0.12 %.

Rapid shallow breathing. Plasma fibrin 0.51 %, globulin 2.78 %, albumin 2.75 %.

Moderate oedema. Died 16. ix. 22.

Infective endocarditis. Moderate fibrosis of kidneys. Slight oedema. No dyspnoea.

Much worse; oedema; hyperpnoea. Plasma fibrin 0.50 %, globulin 0.49 %, albumin 3.39 %.

Morphia given from 27. x. 22. Hyperpnoea much less; twitching.

Died 6. xi. 22. Taken 10 minutes after death.

Chronic interstitial nephritis. Had one fit and died 6. xii. 22.

Extensive fibrosis of kidneys. Conscious but drowsy. Ca in serum 0.010 %.

About 100 g. sodium bicarbonate on 18 and 19. xi. 22. Ca in serum 0.008 %. Died 8. xii. 22.

Chronic parenchymatous nephritis. General oedema.

Chronic parenchymatous nephritis. General oedema.

Much worse; more oedema; dyspnoea. Condition unchanged.

38	12. v. 22	7.4	0.025	0.100	0.0033	0.131	—	—	115	—	Chronic parenchymatous nephritis. General oedema. Given 20 grains sodium bicarbonate 3 times a day from 24. v. 22.
	3. vii. 22	7.4	0.040	0.073	0.003	0.116	0.122	—	96	33	Much worse. More oedema. Serum Ca 0.008%.
	28. vii. 22	7.4	0.038	0.082	0.003	0.123	0.126	0.0735	96	25	Somewhat better.
39	21. vi. 22	7.2	0.0125	0.105	—	—	—	—	150	—	Only kidney removed for pyonephrosis 17. vi. 22.
	22. vi. 22	7.3	0.028	0.102	0.004	0.134	0.130	—	270	—	Given 20 grains sodium bicarbonate 3 times a day from 21. v. 22.
	26. vi. 22	7.35	0.029	—	0.003	—	—	—	330	—	Drowsy during last few days. Post mortem, slight oedema of back; pleural effusion 16 oz.
	29. vi. 22	7.35	0.028	0.066	0.002	0.096	0.117	0.047	450	—	—
40	P.M. 31. vii. 22	—	—	0.058	—	—	0.117	—	510	—	Diabetes, ketosis, necrosis of kidneys, anuria.
	31. vii. 22	7.25	0.014	0.082	0.002	0.098	0.119	0.0633	240	50	Hyperpnoea slight, NaHCO ₃ grains 20 t.d. from 29. vii. 22.
41	23. ii. 22	—	0.046	0.090	—	0.136	0.165	—	130	40	Toxaemia of pregnancy. Treated with enormous doses of sodium bicarbonate. Slow, shallow breathing, coma, tetany, death. Ca in serum 0.010 %.
<i>Hyperpnoea:</i>											
42	—	—	0.031	0.103	(0.002)	0.136	0.135	—	27.5	—	—
43	5. xi. 21	—	0.033	0.102	(0.002)	0.137	0.133	—	33.5	—	—
44	4. i. 22	—	0.036	0.104	0.003	0.137	0.137	—	26.5	—	—
45	3. xii. 21	—	0.036	0.097	(0.002)	0.135	0.133	—	48	—	—
	18. viii. 22	7.4	0.028	0.101	0.002	0.131	0.126	0.0660	54.5	50	Given large doses of sodium bicarbonate.
46	27. vii. 22	7.45	0.030	0.095	0.003	0.128	0.126	—	39	50	No alkali given on this occasion.
47	28. viii. 22	7.55	0.031	0.093	0.002	0.126	0.130	0.075	66	—	—
<i>Heart failure:</i>											
48	14. viii. 22	7.4	0.0335	0.102	0.002	0.137	0.135	—	53	55	Much oedema, cyanosis and dyspnoea.
49	28. vi. 22	7.5	0.026	0.092	0.0015	0.119	0.122	—	43.5	55	Oedema, cyanosis and dyspnoea.
50	22. v. 22	7.35	0.0275	0.0865	0.0035	—	—	—	53	—	Oedema, cyanosis and dyspnoea.
51	9. iii. 22	7.5	0.029	0.1025	—	—	—	—	35	—	Little oedema, no cyanosis, great dyspnoea.
	—	—	—	—	—	—	—	—	—	—	Died of heart failure 25. xii. 22.
52	9. xi. 22	7.6	0.021	0.101	0.002	0.124	0.130	—	63	—	Infective endocarditis. Moderate oedema, cyanosis and dyspnoea.
	10. xi. 22	7.45	0.019	0.096	0.002	0.117	0.126	—	56	30	Worse.
	11. xi. 22	7.45	0.020	—	—	—	—	—	54.5	—	Began to improve 12. xi. 22.
	15. xi. 22	7.45	0.0225	0.105	0.001	0.126	0.133	—	41	30	Much better, no dyspnoea.
	20. xi. 22	7.5	0.029	0.102	0.002	0.133	0.126	—	28	30	" "
<i>No evidence of nephritis:</i>											
53	—	7.4	0.0305	0.102	0.0015	0.134	0.133	—	—	—	Normal woman one day after delivery.
54	18. v. 22	7.35	0.027	0.098	—	0.125	0.126	—	24	—	Renal glycosuria. Serum Ca 0.010 %.
55	13. vii. 22	7.4	0.029	0.097	—	0.126	0.126	—	—	—	" "
56	8. viii. 22	7.5	0.024	0.110	0.002	0.136	0.135	0.003	28	—	Diabetes. No ketosis.
57	—	7.4	0.031	0.095	0.002	0.128	0.126	—	—	—	Normal adult.
58	—	7.4	0.0255	0.105	0.0015	0.132	0.133	—	—	—	" "
59	—	—	0.0235	0.1105	0.0015	0.1355	0.135	—	—	—	Normal adult. Loss of CO ₂ in collecting blood.

SUMMARY AND CONCLUSIONS.

1. $[\text{HCO}_3^-]$, $[\text{Cl}^-]$, $[\text{HPO}_4^{--}]$ and $[\text{Na}^+]$ were determined in plasmata and in a few cerebrospinal fluids of patients with nephritis, also in plasmata and cerebrospinal fluids of control cases.

2. The plasma $[\text{HPO}_4^{--}]$ was increased in the majority of cases with high blood urea.

3. $[\text{Na}^+]$ was not increased in the plasma even in extreme degree of renal inefficiency, on the other hand low values were found in several such cases.

4. Evidence is given of disturbance of the distribution of chlorides between the plasma on the one hand and cerebrospinal fluid and tissues on the other.

5. Very low figures for plasma $[\text{Cl}^-]$ were found in some severe cases of nephritis.

6. The excess of kation in normal plasma unaccounted for by $[\text{HCO}_3^-]$, $[\text{Cl}^-]$, and $[\text{HPO}_4^{--}]$ is combined mainly with protein; a little may be combined with organic anions.

7. In many cases of nephritis with much urea retention an excess of kation over and above that combined with HCO_3^- , Cl^- , HPO_4^{--} and protein was found. This was combined with anions, sulphanion among others, classed as undetermined anions. Such undetermined anions were found in cases with and without acidosis.

8. The p_{H} of the majority of cases lay within normal limits, especially about 7.40. High values up to 7.6 were found, these were caused by disturbances of the respiratory centre and by treatment with sodium bicarbonate.

9. In most cases of acidosis in nephritis, the reduction of plasma $[\text{HCO}_3^-]$ was due to accumulated HPO_4^{--} and undetermined anions; of these, undetermined anions were the more important factor. In several cases the effect of these anions was much reduced by a coincident fall of $[\text{Cl}^-]$. In only one case was there evidence of a specific retention of Cl^- as the cause of the acidosis.

10. Dyspnoea in nephritis is not caused by acidosis but by disturbances of the circulation and respiration. Hyperpnoea in acidosis in nephritis is not striking even in severe cases.

11. Acidosis in nephritis is evidence of a very severe condition but is not harmful in itself. It does not cause any of the symptoms of uraemia.

12. The $[\text{HCO}_3^-]$ of the cerebrospinal fluid is the same as that of the plasma. Reduction of $[\text{HCO}_3^-]$ in cerebrospinal fluid is caused by excess of $[\text{Cl}^-]$.

I take this opportunity to thank the Physicians of the London Hospital for the facilities offered me in the investigation of these cases; also my wife, Miss D. S. Russell and Mr T. Cathrall for the estimation of phenolsulphone-phthalein excretion, of urea in blood and urine, and other assistance.

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XXXI. RATE OF LIBERATION OF ACID BY $\beta\beta'$ -DICHLORODIETHYL SULPHIDE AND ITS ANALOGUES IN ITS RELATION TO THE "ACID" THEORY OF SKIN VESICATION.

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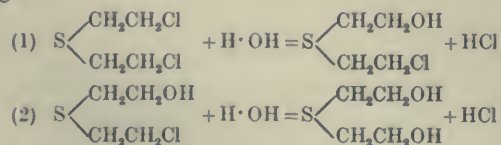
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INTRODUCTION.

THE theory that the intracellular liberation of acid is the effective agent in producing vesication by $\beta\beta'$ -dichlorodiethyl sulphide (described as DES throughout this report) has received support by E. K. Marshall [1919, 1920] and other American workers [Lynch, Smith and Marshall, 1918], though others [Warthin and Weller, 1919] oppose this view. Two ideas seem to be involved, (a) that owing to its ready lipoid solubility, DES easily reaches the inside of the cell, and (b) that in the cell, DES splits off acid at a favourable rate for the production of pathological change. If the theory is correct, analogous lipoid-soluble substances which split off acid either at the same rate or faster than DES should be vesicant, and conversely analogous substances, which split off acid much slower than DES should be either non-vesicant or very much less vesicant than DES. The test of the theory therefore depends upon an investigation of the rate at which acid is split off from DES and analogous compounds under conditions approximating as nearly as possible to the conditions under which DES would break down in the body cell.

Previous work upon the rate of acid liberation by DES has been published. Rona [1921] investigated by a conductivity method the rate of so-called hydrolysis of a series of compounds, including DES and dichlorodimethyl sulphide. His general conclusions were that dichloromethyl sulphide hydrolysed "quickly, but measurably," DES "gradually," tetrachlorodiethyl sulphide "gradually"; DES hydrolysed according to the monomolecular law.

Russell [Report] by the same method concluded that the reaction proceeded in two stages:



though he found difficulty in interpreting some of the data obtained. Other workers have employed titration methods. E. F. Hopkins [1919] concluded

that the rate of liberation of acid proceeded according to the monomolecular law. The method used by him, viz. of withdrawing samples and cooling in mixtures of ice and calcium chloride, gave unreliable results in our hands owing to the difficulty of stopping the reaction during the cooling.

There is evidently some disagreement as to whether the reaction follows the monomolecular law. Some workers have found difficulty in accepting the "acid" theory. Flury and Wieland [1921] for instance suggest in a discussion that oxidation in the cell may play its part in the formation of vesicant substances from DES. In support of this they quote dichlorodiethyl sulphoxide and dichlorodiethyl sulphone, both of which they consider to be vesicant¹. The suggestion that substances formed by the cell from DES may be the actual vesicants is of some importance, if the "acid" theory cannot be accepted. We have investigated the rate at which several of these substances yield acid by the following methods.

METHODS.

Whatever method is adopted, the first point to be settled is that of obtaining a satisfactory solution. Hopkins [1919] by vigorously shaking DES with large quantities of water and subsequently withdrawing the clear supernatant solution, estimated the solubility of DES in water to be of the order of 0.07 % at 10°. Boulin and Simon [1920] obtained a figure of 0.048 %. The solubility is therefore slight. Experimentally we found that when 0.04 % of DES was shaken with water the whole body of the solution was filled initially with minute droplets. An examination of the solution under the microscope even after some minutes, by refracted light, showed the presence of finely suspended droplets, after the solution had become apparently clear to the naked eye. A microscopic examination of two solutions made (1) by shaking DES alone with water, and (2) by adding an alcoholic solution of 0.04 % DES to water made it clear that the alcoholic solution became homogeneous much more rapidly. Accordingly we have used for our experiments solutions of DES made by adding water to DES dissolved in alcohol, the total strength of DES being not more than 0.04 %, and of alcohol usually not more than 5 %. Freshly made, these solutions show a turbidity, which clears upon shaking in the course of a minute.

In the choice of a method of estimating the amount of acid liberated, we have been guided by two considerations, firstly that the acid should be estimated as such, and secondly that the acid should be liberated under conditions approximating to those in the living cell. Recent research has shown that the organism is able to call upon large reserves of alkali when necessary, in fact the reaction of the blood is adjusted with great accuracy in the region of p_{H} 7.4 under wide variation of external condition. This seems to indicate that in the cell the liberation of acid by DES must be accompanied by a neutralisation of the acid formed, at any rate in the initial stages. The body

¹ Marshall considers the "sulphone" to be vesicant, but not the "sulphoxide."

cell will carry out a titration of the acid at constant reaction. In the case of the body cell the extreme limits of reaction will not be greater than p_H 6.0–8.0. The idea of withdrawing samples from a DES solution in which the concentration of hydrogen ions was gradually accumulating was therefore abandoned. Instead, the progress of the reaction was estimated by titrating to a constant, slightly acid reaction. Most experiments were done using methyl-red as indicator, with an end point of p_H 5.3, using CO_2 -free alcoholic soda ($N/100$) as titration solution. By the use of methyl red, errors introduced by the taking up of CO_2 during the experiments were largely avoided. The additions of alkali were kept low (2 cc. of $N/100$ NaOH at a time), so that without the use of buffer solutions the liberation of acid could be studied within the limits of reaction p_H 5.0–9.0 approximately. A final control in the presence of $M/1000$ phosphate in which the reaction could be kept accurately between the limits p_H 7.0–7.4 gave essentially the same course for the acid curves. The rate of acid liberation is obtained complicated only by a slight dilution of the solutions with water and by a gradual accumulation of traces of NaCl in the solution. These factors will be discussed below.

Description of method.

The method finally adopted was as follows. Distilled water, subsequently boiled to drive off CO_2 and cooled with a soda lime tube attached to the containing flask, was used throughout. Two flasks, one containing 95 cc. of CO_2 -free water plus indicator (methyl red) and the other, 5 cc. of absolute alcohol, were placed in the bath and allowed to assume the required temperature. Two drops of DES¹ (0.0384 g.) were then transferred by means of a dry pipette to the flask containing the alcohol. Next the 95 cc. water were poured rapidly into the alcoholic solution, and the stop watch started, the receiving flask being shaken whilst immersed in the water. A clear solution was obtained in about half a minute. After the lapse of a minute (to ensure complete solution) the titration was started, using 5 % alcoholic soda, $N/100$. Alkali was added until the solution, at first pink, went yellow. The burette was then read. The time of return of the colour to pink as compared with a standard flask clamped in the bath was then noted. The water-bath was of white enamel in order to aid the observation of these colour changes. As soon as the time of return was noted, more alkali (1–2 cc.) was added and the observations repeated. This was continued until it was judged by the slowness of the change that the stage of complete hydrolysis was nearly reached. During the whole of these operations, the flask was kept immersed in the water and subjected to gentle shaking. It was possible to observe the return of colour with great accuracy, the time readings being correct to the second. When the reaction was apparently finished, the solution was diluted with distilled CO_2 -free water and heated upon the water-bath. Upon becoming acid more alkali

¹ The DES used was made by the sulphur monochloride method and purified by fractional distillation under reduced pressure.

was added until neutral, this being continued until no more acid was formed, usually 24 hours. When not on the water-bath the flasks were kept tightly stoppered to prevent access of CO_2 . The above method was subject to such variations as the experiments demanded.

The determination of the end point, that is the maximum amount of acid which could be liberated, gave a certain trouble. Other workers have used Volhard's method for the total chloride. This was tried, and also Larrson's modification of Volhard's method, but both were rejected owing to the indefiniteness of the end point. This may be due to the interference of thiodiglycol in silver titrations discussed by the French workers [Moureu and Murat, 1920]. The method described above was finally adopted as giving the most satisfactory results. It must be noted that under these conditions there is no strict equilibrium point. The DES splits down smoothly, until all of it has disappeared. This was judged both from the titration values and also by skin tests. A number of solutions, in which hydrolysis was considered to be complete by titration values, were tested on the skin of the forearm for blistering power. In all cases a negative result was obtained.

The validity of the method.

In the case of DES we are not dealing with a case of saponification by alkali of the ordinary type. Liberation of HCl by the action of water proceeds at a markedly acid reaction in the absence of alkali. It is only slightly inhibited in the presence of $N/100$ HCl (at a $p_H = 2.0$). The addition of NaOH therefore does not initiate this reaction. Hence in a justification of the method, we are concerned with showing

- (a) what is the effect upon the course of the reaction of the addition of small amounts of alkali;
- (b) what is the effect of allowing the reaction to proceed in the presence of accumulating acid;
- (c) what is the influence of the indicator, if any;
- (d) what disturbance is introduced by the presence of accumulating NaCl.

The effect of dilution can be left out of account as the solution initially contains 2000 times more water than DES.

(a) The effect of alkali.

Fig. 1 is a curve made from three separate experiments, in which the percentage acid liberated is plotted against the time. It will be seen that the observations lie quite smoothly; in itself this constitutes indirect evidence that the addition of alkali has not appreciably disturbed the course of the reaction, because it is impossible to add accurately the same amounts of alkali at each stage, owing to the speed at which the reaction is proceeding. We tried to find any accelerating effect of alkali by carrying out the experiments in the presence of 15–20 cc. of alkali added initially. Points marked "alkali"

points in Fig. 1 were obtained in this way. It will be seen that the presence of 5–10 times as much alkali as is normally present throughout a considerable part of the reaction only leads to small increases in the amount of acid liberated. The largest increase amounts to some 3 %. This makes it evident that upon the alkaline side of the neutral point the rate of acid liberation is remarkably independent of p_{H} . The p_{H} in these experiments must have been more alkaline than p_{H} 11.0 during part of the reaction.

The details of the experiment are shown in Experiment 1.

Exp. 1. A 300 cc. solution was made up as previously described. Immediately after mixing, portions of 100 cc. measured roughly were decanted into two flasks, the first containing 15 cc. $N/100$ NaOH and the second 20 cc. $N/100$ NaOH. The original flask in which about 100 cc. solution remained was titrated in the standard manner. The two flasks containing alkali initially were kept under observation and the time of return of colour from yellow to pink noted. All three flasks were kept immersed in the same water-bath throughout.

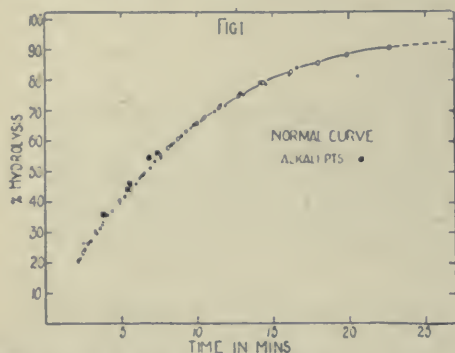


Fig. 1. DES in 5 % alcoholic solution. Ordinates % acid liberated. Abscissae Time. Temp. 24.5 ± 0.10 . Points \bullet \odot $+$ represent three different experiments. Points marked \blacksquare obtained in the presence of alkali.

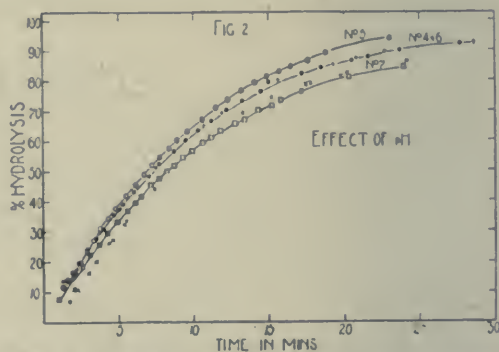


Fig. 2. Reaction of titration end point varied. Temp. $24.6^\circ \pm 0.1$. Curves: No. 4, p_{H} 5.3 (control). No. 5, p_{H} 9.0. No. 6, p_{H} 7.1. No. 7, p_{H} 9.0 exposed specimen of DES. X, p_{H} 7.0 (in presence of phosphate $M/1000$).

Confirmation of this unexpected behaviour to alkali was obtained by performing titrations to end points of varying reaction, using indicators other than methyl-red. In the case of the experiments with thymol-blue (end-point p_{H} 9.0), especial care was taken to keep the reaction alkaline throughout, the fresh alkali being added at the instant of the return of colour. Fig. 2 shows the curves obtained. Nos. 4 and 6 were at p_{H} 5.3 and 7.1 respectively. Nos. 5 and 7 were both at p_{H} 9.0, No. 5 is slightly accelerated as compared with Nos. 4 and 6. No. 7 shows the greatest divergence from its fellow No. 5. It is included here in order to introduce a puzzling irregularity which we have encountered, in specimens of DES which have been repeatedly exposed to the air. We have found a tendency to a slowed rate of acid liberation in such specimens, which cannot be accounted for by the supposition of preformed acid.

The points marked X in Fig. 2 were obtained at a reaction p_{H} 7.0–7.4 by

using traces of phosphate as buffer ($M/1000$). It is evident that there is no significant difference from the other curves.

(b) *The effect of acid.*

Fig. 3 shows the effect upon the course of the reaction of (a) added acid and (b) of the maintenance of an acid reaction of p_H 3.6 approximately. The details of the experiment were as follows:

Exp. 2. 400 cc. solution were made, containing the usual 0.04 % DES and 5 % alcohol. Immediately upon solution, portions of about 100 cc. were rapidly decanted into three flasks, two of which contained 15 and 20 cc. of $N/100$ HCl respectively. All four flasks were left in the water-bath. At intervals the flasks were titrated. The end points were obtained in the usual way, allowing for the presence of the added acid where necessary.

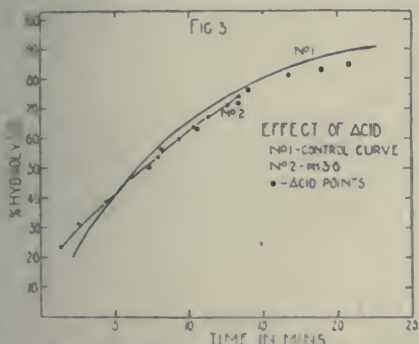


Fig. 3. Effect of acid. Ordinates and abscissae as before. Curve 1. Temp. $24.5^\circ \pm 0.1^\circ$. Curve 2. Temp. 24.6° .

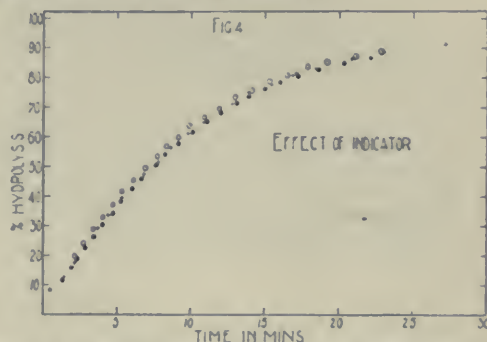


Fig. 4. Ordinates and abscissae as before. Temp. $24.6^\circ \pm 0.1$. Effect of indicator and small increase in alcohol. 1. Normal curve from Fig. 1, ●. 2. Twice normal amount of indicator, ○. 3. Normal amount of indicator plus 6 % alcohol, +.

The points obtained from the flasks containing initial acid are seen to lie well off the control curve. This slowing effect of acid has been noted by other observers. It is brought out in the experiment conducted at a reaction of p_H 3.6, with bromphenol blue as indicator, though the latter experiment was not satisfactory owing to the difficulty of obtaining readings of the colour change. Under the conditions of the method, where the flasks are only allowed to go acid momentarily, this slowing effect of acid is avoided as a complication of the results.

(c) *The effect of indicator.*

With one or two exceptions the indicator used throughout was methyl-red. This indicator is made up in 60 % alcoholic solution and the amount used in any experiment was of the order of 0.7 %, containing 0.00014 g. methyl-red. As no allowance was made for the extra alcohol added in this way, the effect of doubling the amount of indicator by weight was tried, as also that of increasing the strength of alcohol to 6 % instead of 5 %. The results are shown diagrammatically in Fig. 4. We may conclude that the indicator added had no effect upon the course of the reaction.

(d) Effect of NaCl formed during the progress of the reaction.

There is a slight effect produced by the accumulating NaCl which will be discussed later. In the early stage of the reaction, up to 60 % hydrolysis¹, the effect is slight.

From the above it will be seen that the precautions to be adopted in using the method are (1) to add the alkali in small amounts at a time and (2) not to allow the solutions to remain acid at any stage.

We have compared by this method the rate of liberation of acid from DES in water solutions only, in mixtures of water and alcohol, at various temperatures, in the presence of salts, and in the presence of a few substances of physiological interest.

THE COURSE OF THE REACTION.

Table I is the record of a typical experiment in 5 % alcohol; k is calculated from the equation

$$k = \frac{1}{t_1 - t_0} \log_e \frac{a - x_0}{a - x_1},$$

t refers to time and x to % acid liberated. t_0 and x_0 are taken at a convenient point between 1 and 2 minutes after the beginning of the reaction, in order to avoid the influence of irregularities at the start, or of traces of preformed acid in the specimen of DES.

Table I. *Temp.* 24.6°.

Time	% A	k	Time	% A	k
1' 18"	12.6	—	10' 19"	63.7	.099
1' 54"	16.7	—	11' 10"	66.8	.100
2' 19"	24.3	.094	12' 14"	70.3	.100
3' 30"	27.9	.090	13' 20"	73.4	.100
3' 57"	31.0	.092	14' 30"	76.6	.101
4' 43"	35.5	.091	14' 59"	79.7	.109
5' 15"	39.1	.094	17' 10"	82.1	.101
6' 2"	43.3	.094	18' 30"	84.2	.101
6' 57"	48.5	.096	20' 30"	86.8	.099
7' 43"	52.5	.097	21' 35"	87.9	.098
8' 41"	56.7	.097	23' 42"	90.0	.097
9' 29"	60.4	.099	27' 40"	92.4	.093

Here, and throughout, A refers to the amount (percentage) of acid liberated at the time stated and is calculated upon the basis of total amount of acid liberated as determined by the final titration.

k calculated upon a monomolecular basis, increases slightly during the stage 28–82 % hydrolysis and then falls to 90 % hydrolysis, the total variation being 0.090 to 0.101, that is about 11 %. In general it may be said that the reaction simulates a monomolecular reaction under these conditions, although over no range is k absolutely constant. There is a steady rise to 80 % hydrolysis, a rise which is masked perhaps slightly by the presence of accumulating NaCl in the solution. The extent of this effect will be considered later. For con-

¹ The term "hydrolysis" may not be strictly correct. It is used for convenience to represent "liberation of acid."

venience of comparison, the reactions can be compared by interpolating from the curves the values of k at 30, 40, 50, 60, 70, 80 % acid liberated. Table II gives the results of a series of experiments with 0.04 % DES in the presence of 5 % alcohol.

Table II.

Exps.	k						Remarks
	30% A	40% A	50% A	60% A	70% A	80% A	
1	·093	·096	·100	·102	·102	·102	No phosphate. Reaction p_H 5.0–9.0. Temp. $24.6 \pm 1^\circ$
2	·091	·093	·094	·096	·098	·098	" "
3	·091	·093	·095	·097	·098	·098	" "
4	·090	·092	·094	·096	·097	·096	" "
5	·093	·095	·100	·100	·100	·099	No phosphate. Indicator twice the normal amount
6	·092	·094	·095	·097	·098	·098	No phosphate. 6 % alcohol instead of 5 %
7	·090	·093	·094	·095	·094	·092	Phosphate $M/1000$. p_H 7.2–7.4
8	·084	·088	·093	·094	·095	·090	" "

The reaction seems to be most stable at 50–60 % hydrolysis, and we may take the mean of the 50 and 60 % constants as a characteristic constant χ of any particular reaction. In Experiments 1–6 this becomes 0.097, for experiments 7 and 8 0.094, slightly lower.

The effect of different conditions upon the course of the reaction may be judged (1) from changes in the characteristic constant, and (2) from changes in the curve relating the value of the constant χ and the % acid liberated.

Water only.

There is no appreciable difference between Exp. 1, Table III, with three drops of DES % and Exp. 2 with one drop of DES %; this shows that within these limits, variations in concentration of DES do not affect the % liberation of acid in a given time. $\chi = 0.105$, *i.e.* some 8 % higher than in the case of 5 % alcohol. It is difficult to decide whether this is actually a slowing effect of the alcohol or whether it is due to some solubility question. As t_0 and x_0 were taken some time after the beginning of the reaction, the irregularity due to slow solution should be excluded.

Water and alcohol.

Fig. 5 and Table III, Exps. 5, 6, 7, 8 give the results. Alcohol slows the reaction, χ for 25 % = 0.090 and for 50 %, $\chi = 0.163$. The constant at 25 % behaves as that of a true monomolecular reaction throughout the range, whereas with 50 % alcohol k increases 60 % between 30 and 80 % hydrolysis. This shows that alcohol is also affecting the course of the reaction.

Temperature.

Fig. 6 and Table III, Exps. 6, 9, 10 show the results. The characteristic constant χ is 0.28 at 14.5° , $\chi = 0.097$ at 24.6° and 0.385 at 36.8° . This makes the temperature coefficient between 14.5 – $24.7 = 3.34$, and between 24.7 % and 37.0 % = 3.22, the average being 3.28 per 10° rise in temperature. It is to be noted that at 37° the reaction behaved almost strictly throughout as a monomolecular reaction.

Table III. *Course of reaction under varying conditions.*

Group	Exps.	k						Alcohol	Remarks
		30% A	40% A	50% A	60% A	70% A	80% A		
Water	1	—	·161	·103	·106	·107	·108	nil	3 drops DES %
	2	·089	·101	·104	·106	·106	·104	„	1 drop DES %
	3	—	·107	·105	·107	·108	·108	„	2 drops DES %
	4	·029	·030	·031	·031	·031	·031	„	Temp. 14·7°
Alcohol	5	·093	·097	·101	·103	·107	·112	1	Temp. 24·8° ± 4° (Fig. 5, No. 2)
	6	·091	·093	·094	·096	·098	·098	5	—
	7	·090	·0895	·089	·0905	·091	·091	25	Temp. 24·7° (Fig. 5, No. 4)
	8	·013	—	·016	·017	·018	·019	50	Temp. 24·4°
Temperature	9	·027	·028	·028	·029	·030	·031	5	Temp. 14·5°
	10	—	—	·38	·39	·385	·39	„	Temp. 36·8°
NaCl	11	·095	·091	·094	·095	·095	·093	„	·01 % NaCl in M/1000 phosphate
	12*	·088	·083	·083	·086	·083	·080	„	·05 % in M/1000 phosphate
	13	·066	·069	·070	·071	·072	·072	„	Temp. 24·4°. 0·1 % in M/1000 phosphate
	14	·028	·029	·031	·032	·033	·033	„	Temp. 24·5°. 1 % in M/1000 phosphate
Other salts	15	·010	·011	·012	·012	—	—	„	Temp. 24·7°. 5 % in M/1000 phosphate
	16	·077	·077	·077	·075	·070	·038 (83 %)	„	Temp. 25·4°. 1 % Na ₂ SO ₄
	17	·063	·062	·059	·051	·033 (68 %)	—	„	Temp. 24·8°. 1 % MgSO ₄
Other substances	18	·094	·095	·097	·098	·098	·098	„	Temp. 24·5°. 1 % NaNO ₃ in M/1000 phosphate
	19	·077	·078	·080	·082	·082	·081	„	·1 % diethylamine
	20	·089	·090	·094	·096	·097	·096	„	·036 % lecithin
	21	—	·081	—	·087	·088	·090	„	·1 % glycine hydrochloride
	22	·026	·027	·029	·030	·031	·031	„	Ringer's solution instead of water

The temperature can be relied upon to $\pm 0.1^\circ$. Temp. 24·6° unless otherwise stated.

* The controls to this experiment are Table II, Exps. 7 and 8.

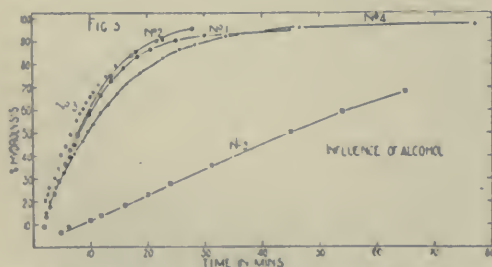


Fig. 5. Influence of alcohol. Ordinates and abscissae as before. Temp. 24·5° ± 0·1°. Curves: No. 1, no alcohol ●. No. 2, 1 % alcohol ○. No. 3, 5 % alcohol *. No. 4, 24 % alcohol +. No. 5, 50 % alcohol □.

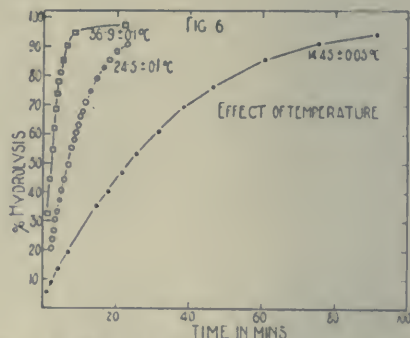


Fig. 6. Effect of temperature. Ordinates and abscissae as before.

Salts.

Fig. 7 and Table III, Exps. 11–18 inclusive show the results. 1 % NaCl changes χ at 24·6° from ·097 to ·028, practically the equivalent of 10° fall in temperature. 0·1 % NaCl in the presence of phosphate, reduced χ from ·094 to ·070, a reduction of 20 %. It is to be noted that the course of the

reaction is not changed, there is still the gradual increase in k , which is found in the absence of added NaCl.

The effect of sulphates is remarkable, up to 50 % hydrolysis very little change is noted in the course of the reaction, but after 50 % the reaction slows rather abruptly, and proceeds to completion only after the lapse of several days. We have not attempted to investigate the cause of this remarkable effect of sulphate.

Sodium nitrate (1 %), it will be seen, has no effect upon the reaction.

Some other substances.

Table III, Exps. 19, 20, 21, 22 show the effect of diethylamine, lecithin, glycine hydrochloride and Ringer's solution. The slight retardation observed with glycine and diethylamine is undoubtedly due to the effect of chlorides. Ringer's solution has the effect which can be expected from its salt content.

Summary of Results.

Summarising the above data, the reaction from the broad standpoint resembles a monomolecular reaction. It is apparent however that we may be dealing with a balance of factors, masking a more complicated reaction for the following reasons:

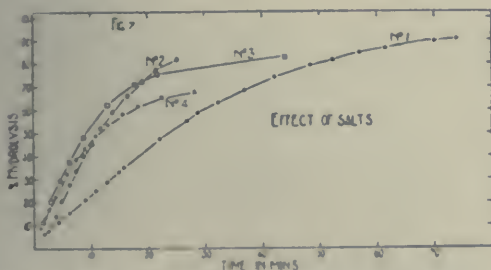


Fig. 7. Effect of salts. Ordinates and abscissae as before. Curves: No. 1, 1 % NaCl. No. 2, 0.1 % NaCl (in presence of $M/1000$ phosphate). The controls to this curve are given in Fig. 2. No. 3, 1 % Na_2SO_4 . No. 4, 1 % MgSO_4 . Temp. Nos. 1, 2, 4, $24.5 \pm 0.1^\circ$. No. 3, 25.4° .

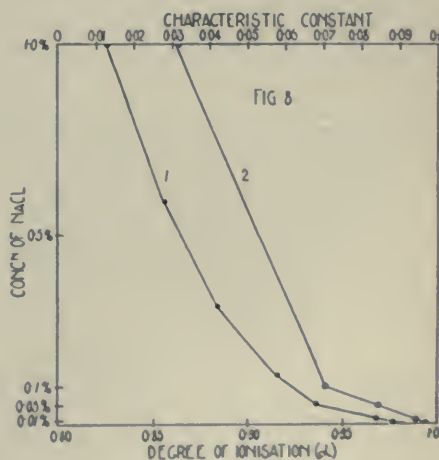


Fig. 8.

(1) There is usually a definite increase in the constant k as the reaction proceeds from 30–80 % hydrolysis. This increase found under the conditions of experiment used is smaller than would be observed in the absence of accumulating NaCl. If we correct the data of Exp. 2, Table II by extrapolation for the amount of NaCl retardation at different stages from the known data from Exps. 11–15, we obtain the following figures, Table IV.

Table IV.

	<i>k</i>					
	30 % A	40 % A	50 % A	60 % A	70 % A	80 % A
Uncorrected	·091	·093	·094	·096	·098	·098
Corrected	·093	·096	·098	·100	·103	·104

It is not pretended that these corrected figures are more than an approximation to the truth, but they tend to confirm the opinion that the action of water upon DES is not absolutely simple.

(2) The course of the reaction is not always the same quite apart from any slowing effect. Sometimes as in the case of 25 % alcohol at 24·6°, and with 5 % alcohol at 37·0° the reaction behaved almost strictly as a monomolecular reaction. With 50 % alcohol, there is a much more rapid acceleration than normal as the reaction proceeds. With sodium sulphate the reaction is completely changed. This would receive a simple explanation if the reaction went in two stages, one of which was inhibited by sulphate. The subject will be discussed below.

Meanwhile one generalisation seems clear. Conditions which influence the reaction all slow the rate at which DES yields acid. In the cell therefore in which we have a concentration of some 0·5 % NaCl, the rate of liberation of acid will be slow. It will be further slowed by the removal of DES from the aqueous part of the cell by solution in lipid phases. In itself this militates against the "acid" theory of vesication.

The next section deals with the behaviour of analogous compounds.

ANALOGUES OF DES.

The following were placed at our disposal: $\alpha\alpha'$ -dichlorodimethyl sulphide, $\alpha\beta\beta'$ -trichlorodiethyl sulphide, $\alpha\beta\beta\beta'$ -tetrachlorodiethyl sulphide and $\alpha\alpha\beta\beta\beta\beta'$ -hexachlorodiethyl sulphide. Of these the first named liberated acid very readily in contact with water, and the remainder only with difficulty. In the case of dichlorodimethyl sulphide, a clear homogeneous solution was obtained under the same conditions as with DES. Table V gives the details of three experiments.

Table V. *Dichlorodimethyl sulphide.*

	<i>k</i>						
Exps.	30 % A	40 % A	50 % A	60 % A	70 % A	80 % A	Remarks
1	·16	·16	·15	·15	·15	·15	Temp. 14·7°
2	—	·42	—	·40	·40	·40	Temp. 24·6°. <i>M</i> /1000 phosphate
3	—	·47	—	·47	—	·44	Temp. 24·6°. <i>M</i> /1000 phosphate, NaCl %

Acid is liberated from dichlorodimethyl sulphide at a markedly faster rate at 14·7° than from DES at 24·6°, the velocity constant at the same temperature being some five times greater. Further it must be noted that the presence of 1 % NaCl does not slow this reaction, thereby indicating a difference of chemical behaviour between the two compounds.

There is another respect in which the compound differs from DES. During

the progress of the reaction, a fine white precipitate¹ gradually accumulates; this is not sufficient to interfere with the colour observations. The end point is reached almost immediately after the observations are discontinued, there is no need to heat or to allow the mixture to stand for some time. The insolubility of the precipitate probably removes the hydrolytic product from the sphere of action, so allowing the reaction to go quickly to completion. This is concordant with the steady fall observed in the constant.

With regard to the other three compounds, it is difficult to get reliable data. They were all much less soluble in water than DES. 5 % alcohol failed to take them into solution. 25 % did not ensure complete solution except in the case of the trichloro derivative. It seemed essential to obtain a homogeneous solution before any measurements were attempted, therefore 50 % alcoholic solutions were used. Under these conditions complete solution was assured, but even at 38° liberation of acid was slow. Relative measurements were obtained finally at 52°. These indicated that under the prescribed conditions, acid is liberated most readily from the hexachloro compound (50 % in 70 minutes), and least readily from the trichloro compound (no apparent hydrolysis). The tetrachloro compound occupies an intermediate position (50 % hydrolysis in 100 minutes). Under the same conditions (50 % alcohol solution), calculating from the results at 25°, DES should liberate 50 % of its acid in three minutes. k for the hexachloro derivative is shown in the following table, it falls throughout the reaction.

k is here calculated according to the equation

$$k = \frac{1}{t} \log_e \frac{a}{a-x}.$$

Table VI. *Hexachlorodiethyl sulphide.*

% A	k	% A	k
4.5	.021	34.3	.016
8.6	.021	38.4	.015
13.8	.022	42.6	.013
19.6	.023	45.7	.011
24.1	.021	49.0	.010
29.2	.020		

Dichlorodiethyl sulphone.

The following experiments, Table VII, show that dichlorodiethyl sulphone hydrolyses about 100 times less rapidly than DES at 37°, and that NaCl has a depressant action upon it similar to that upon DES.

Table VII.

Exp. 1. Temp. 37.0°			Exp. 2. Temp. 37.2° 1 % NaCl			Exp. 3. Temp. 24.6° 1 % NaCl		
Time	% A	k	Time	% A	k	Time	% A	k
34'	10.7	—	40'	5.1	.0013	100'	3.7	.00035
57'	18.8	.0042	64'	10.3	.0017	244'	10.2	.00041
109'	27.6	.0046	93'	17.4	.0020	—	—	—
135'	33.8	.0037	163'	25.3	.0018	—	—	—
316'	48.3	.0002	—	—	—	—	—	—

¹ This substance was not identified, but it has many of the properties of diethylene disulphide.

In each experiment the strength was the same, viz. 0.04 g. "sulphone," 5 cc. alcohol, 95 cc. $M/1000$ phosphate. Reaction p_H 7.0–7.4. In Exps. 2 and 3, k is calculated according to the equation $k = \frac{1}{t} \log_e \frac{a}{a-x}$. In these two experiments (where 1 % NaCl was present) it was impossible to obtain the end-point by the usual method. Even after three days, during which the flasks were kept at a temperature of 50–60°, the liberation of acid had only attained 50 % of the theoretical yield. The solutions, however, still retained their vesicant properties. The final reading was therefore obtained by calculating the yield of HCl from 0.04 g. of "sulphone." All three experiments can only approximate in accuracy to our experiments with DES, owing to the extreme slowness of the reaction. There is a tendency for k to fall off as the reaction proceeds.

DISCUSSION.

The acid theory of vesicant action.

If we compare the relative rate of acid production, at the same temperature reckoning DES as 100, the following approximate rates may be assigned:

A.	$\beta\beta'$ -dichlorodiethyl sulphide	100	Vesicant
B.	$\alpha\alpha'$ -dichlorodimethyl sulphide	500	Non-vesicant
C.	$\alpha\beta\beta'$ -trichlorodiethyl sulphide	nil	,,
D.	$\alpha\beta\beta\beta'$ -tetrachlorodiethyl sulphide	4	,,
E.	$\alpha\alpha\beta\beta\beta\beta'$ -hexachlorodiethyl sulphide	1	,,
F.	$\beta\beta'$ -dichlorodiethyl sulphone	1	Vesicant

Of this list only *A* and *F* are vesicant. All are soluble in both water and lipid solvents. As *B* and *C* split off acid respectively faster and slower than DES *in vitro*, and are also non-vesicant, it is unlikely that their behaviour will be different in the cell. In this case, if acid liberation caused vesication, dichlorodimethyl sulphide should be a very powerful blisterer. It is non-vesicant. Turning to the other side of the picture, we have DES and its sulphone, of which the latter splits off acid about 100 times more slowly than DES. Both are vesicant, though the sulphone is less so than the sulphide¹. It is evident that even if the production of acid has some adjuvant effect in vesication, we must look to some other property for the true causative factor. Further if we argue from the slowing effect of NaCl upon dichlorodiethyl sulphone *in vitro*, we are forced to conclude that in the cell the "sulphone" does not split off acid at all, and yet it is definitely vesicant. We think therefore that the "acid" theory must be dismissed.

The fact that a concentration of sodium chloride of 1 % practically inhibits the breakdown of dichlorodiethyl sulphone, as well as affording the strongest evidence against the acid theory of vesication, suggests that it is

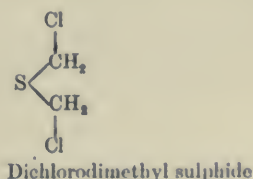
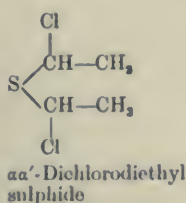
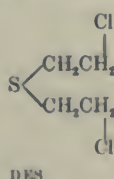
¹ Dr Dixon and Mr Gooding have told us in conversation that they have found the "sulphone" rather less vesicant than the "sulphide."

the chlorine-containing compound itself in the non-hydrolysed condition, which is responsible for the vesicant action. The experiments of Padtberg [1910] and others are of interest in this connection, because they have shown that (1) the skin is among the parts of the body which contain the greatest concentration of chloride, and (2) that the chloride concentration of the skin can be raised by drinking NaCl, the skin acting as Cl depot.

Introduction of Cl atoms into DES results in substances which do not readily liberate acid and which are non-vesicant. The acid theory has been dismissed. What other effect does the introduction of Cl atoms have which renders the compounds non-vesicant? Mann and Pope [1922] state that as the number of Cl atoms in the molecule of DES increases the S atom shows increasing reluctance to become quadrivalent and (in particular) that the facility with which the sulphoxide is formed diminishes. In short the chemical activity of the S atom is shut down. When the S atom in DES is replaced by O the resulting compound $\beta\beta'$ -dichlorodiethyl ether is non-vesicant. Finally it may be recalled that allyl isothiocyanate (C_3H_5NCS) produces blisters when in contact with the skin. The only relation which this compound holds to DES is that it contains a sulphur atom.

The above evidence seems to indicate again that it is the unhydrolysed molecule which is the vesicant agent, and that the presence of a reactive "S" atom has an important bearing upon the vesicant power of these substances.

As an illustration of the complexity of the problem, it may be mentioned that $\alpha\alpha'$ -dichlorodiethyl sulphide is devoid of vesicant properties. An inspection of the formula, however, will show that this compound bears a closer resemblance in structure to the non-vesicant dichlorodimethyl sulphide than to DES. The chlorine atoms are in the same position relative to the S atom in both these compounds.

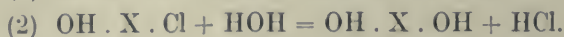
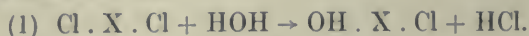


SOME CHEMICAL CONSIDERATIONS.

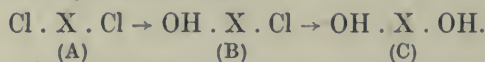
By analogy with esters of the dibasic acids, the hydrolysis of DES would be expected to proceed in two stages. There is actually chemical evidence of some difference between the chlorine atoms of the DES molecule; for instance Mann and Pope [1922] have shown that di-, tri-, tetra- and hexachloro-diethyl sulphide all contain the same group— $\text{S} \cdot \text{CH}_2 \cdot \text{CH}_2\text{Cl}$, i.e. upon chlorinating DES the additional Cl atoms all enter the same half of the molecule.

Russell [Report] has interpreted his results upon the basis of a two stage reaction. In a recent paper (published since the conclusion of this work)

Wilson, Fuller and Schur [1922] claim to have established the case for the two stage reaction; they further think that the second stage proceeds at least five times as fast as the first. Our results show that monomolecular " k " is not constant, and we think that they can only be interpreted upon the basis of a two stage reaction, viz.:



We do not propose to attempt a derivation of kinetic formulae for the two stage reaction, a problem which can be more satisfactorily solved by the isolation and investigation of the intermediate compound. In our experiments chloride slowed the rate of acid liberation, within moderate limits OH-ion did not increase the rate, whereas increase in H-ion (produced by HCl) apparently slowed the rate. We have not investigated how far this effect is really due to increase in H-ion, or is an effect of increasing Cl-ion. Our method of experiment was not accurate at highly acid reactions. Since in our experiments the reaction and therefore the ionic environment has been kept constant, as regards the H- and OH-ions, we have to consider three essential stages, and the effect of Cl-ion upon them, viz.:



The driving force of the reaction may be considered to be derived from the difference in dissociation level between these three compounds. Since the reaction goes to completion, and under these conditions there is no recombination of "C" with HCl, compound "C" represents zero dissociation, compound "B" is intermediate between "A" and "C." Compound "B" in the main governs the rate of the reaction. The dissociation constant for this compound may be represented

$$k_1 = \frac{[\text{X} \begin{smallmatrix} \text{OH}^* \\ \diagdown \end{smallmatrix}] [\text{Cl}']}{[\text{X} \begin{smallmatrix} \text{OH} \\ \diagdown \\ \text{Cl} \end{smallmatrix}]}.$$

It will be seen that k_1 is unaffected by H^* and OH' . OH-ions are only important in so far as the stage $\text{X} \begin{smallmatrix} \text{OH} \\ \diagdown \end{smallmatrix} \rightarrow \text{X} \begin{smallmatrix} \text{OH} \\ \diagdown \\ \text{OH} \end{smallmatrix}$ necessitates a sufficient supply of OH-ion. The dissociation of $\text{X} \begin{smallmatrix} \text{OH} \\ \diagdown \\ \text{Cl} \end{smallmatrix}$ into the ions $\text{X} \begin{smallmatrix} \text{OH}^* \\ \diagdown \end{smallmatrix}$ and Cl' will be sensitive to Cl' ; increasing Cl' will depress the dissociation of the intermediate compound, and will therefore act as a brake or chemical resistance upon the course of the reaction at this stage. This factor will also operate to a certain extent upon the dissociation of the first Cl atom from compound "A." There is left undiscussed the interesting problem as to why, out of the total number of DES molecules present, only a limited number are apparently undergoing change at a given instant. When the reaction is considered from the ionic

standpoint, the absence of depressant effect by NaNO_3 is explained. It is not however clear why Na_2SO_4 and MgSO_4 exerted such a depressant effect upon the later stages of the reaction, a fact which suggests that the sulphate affects only the second stage.

Since the chloride effect seems to be ionic in origin, it is pertinent to enquire whether the depressant effect of NaCl upon the course of the reaction as a whole with increasing concentrations is additive, or whether it follows the dissociation curve for NaCl solutions, obtained from conductivity data. Fig. 8 shows side by side the degree of depression in the characteristic constant produced by NaCl and the degree of dissociation of NaCl as judged from conductivity data (from Lewis). It will be seen that there is close correspondence, suggesting that the ionised part of the NaCl present is responsible for the depressant effect. We find this agreement difficult to reconcile with the theory of the complete dissociation of strong electrolytes (Bjerrum, quoted by [Warburg, 1922]).

It is also puzzling to find that Cl' does not interfere with the rate of reaction of dichlorodimethyl sulphide.

CHEMICAL CONSTITUTION AND VESICANT ACTION.

In connection with attempts to correlate chemical constitution and vesicant power, we feel that it is important to distinguish between (a) an initial disturbance to sensitive structures in the skin, and (b) the subsequent pathological changes. Many substances, unrelated chemically, such as cantharides, strong acids and radium will produce skin burns. Various initial stimuli therefore produce the final result of burn or blister. We feel that the attempt to relate chemical constitution and vesicant action can only be fruitful, if we consider groups of substances having the same initial effect. In a search for the mechanism by which DES irritates the skin, we are trying to find what common factor (chemical or physical) underlies the effect of the mustard gas group of vesicants; this is apparently not acid liberated in the cell. We do not think that it is a surface effect, because DES is not toxic to the protozoon *Colpidium*. Further the view that the "sulphide" is converted into the more stable "sulphone" by the action of skin oxidases [Flury and Wieland, 1921] is not supported by the greater activity of the "sulphide," or by the fact that the "sulphoxide" is non-vesicant. Rona and Petow [1920] claim that the "sulphide" and "sulphone" inhibit the action of urease, but they have also shown that the tetrachlorodiethyl sulphide, a non-vesicant, has a greater effect than the "sulphide." We are left with the possibilities that either (a) synthetic toxic products, or (b) breakdown toxic products are the effective mechanism initiating pathological change.

SUMMARY.

1. The theory that the intracellular liberation of acid is responsible for the vesicant action of dichlorodiethyl sulphide (DES), has been tested by studying the rates of acid liberation by the action of water upon DES and analogous compounds at constant reaction.

2. The rates of acid liberation in 5 % alcoholic solution, were (DES = 100), dichlorodimethyl sulphide 500, DES 100, tetrachlorodiethyl sulphide 4, hexachlorodiethyl sulphide 1, dichlorodiethyl sulphone 1, trichlorodiethyl sulphide 0.

3. In the case of DES, increasing concentrations of alcohol and of NaCl decrease the rate of acid liberation at constant temperature. The presence of NaCl does not slow the rate of acid liberation by dichlorodimethyl sulphide.

4. 1 % NaNO_3 does not influence the rate of acid liberation by DES. Sodium sulphate and magnesium sulphate inhibit the rate strongly in the later stages of the reaction.

5. Between 15° and 38° , the temperature coefficient of the reaction is 3.3 per 10° rise in temperature.

6. The velocity constant, k , of the reaction, calculated upon a monomolecular basis, shows generally a small but steady rise from 30–80 % hydrolysis. This rise is affected variously by different experimental conditions. The conclusion is drawn that the reaction is really a 2-stage reaction, as has been stated by other observers.

7. The acid theory is rendered untenable by the fact that vesicant action and rate of acid liberation do not run parallel in the series of compounds studied; any slight differences in lipid solubility cannot be sufficient to counterbalance the large differences found in the rate of acid liberation.

We are indebted to Professor Sir Wm. Pope for the supply of the compounds used in this research. We are also indebted to Professor Hopkins for valuable discussion.

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XXXII. THE REVERSIBILITY OF THE ACTION OF UREASE OF SOY BEAN.

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(Received March 19th, 1923.)

SINCE Takeuchi in 1909 discovered that the soy bean contained urease in an easily available and very reactive form, many investigations have been made into the question of the mechanism of the hydrolysis of urea to ammonium carbonate by this enzyme, and several attempts have been made, on the basis of the now widely accepted view that the same enzyme which provokes hydrolysis must also produce synthesis, to show that urease is no exception to this general rule.

Specific reversibility of enzyme action, although shown to take place in the case of lipase by Kastle and Loevenhart [1900], Dietz [1907], Armstrong and Gosney [1914], and in the case of emulsin by Bayliss [1912], Bourquelot and Bridel [1913], is by no means so definitely established in the case of most other enzymes. Thus the synthetic action of invertase and the production of maltose from glucose by maltase, to mention only two cases in which specific reversibility has been claimed, are still matters of controversy. Any new direct evidence of specific synthetic power is therefore of considerable value.

Barendrecht [1920] in the course of a long investigation on the application of his radiation hypothesis to the case of urease, stated that this enzyme was reversible, adducing as his evidence that when soy bean meal is added to the first of two equal quantities of ammonium carbonate solution, less ammonia is given off from the former on treatment with potassium carbonate and aspiration through sulphuric acid. If more soy bean meal is added the shortage of ammonia increases, being roughly proportional to the amount of added meal. The actual difference was small, 2-4 parts per thousand of carbonate used. Urea was not isolated. Mattnar [1920] strongly criticised Barendrecht's conclusion, and using Fosse's xanthhydrol reaction for urea showed that urea was not produced under the conditions described by Barendrecht even to the one-fortieth part of the small extent that the latter had supposed. It was evident that if any direct synthesis of urea by this enzyme took place at all, the position of equilibrium was very near complete hydrolysis.

Fearon [1923] has detected and isolated cyanates in the reaction mixtures during the decomposition of an aqueous solution of urea by soy bean urease,

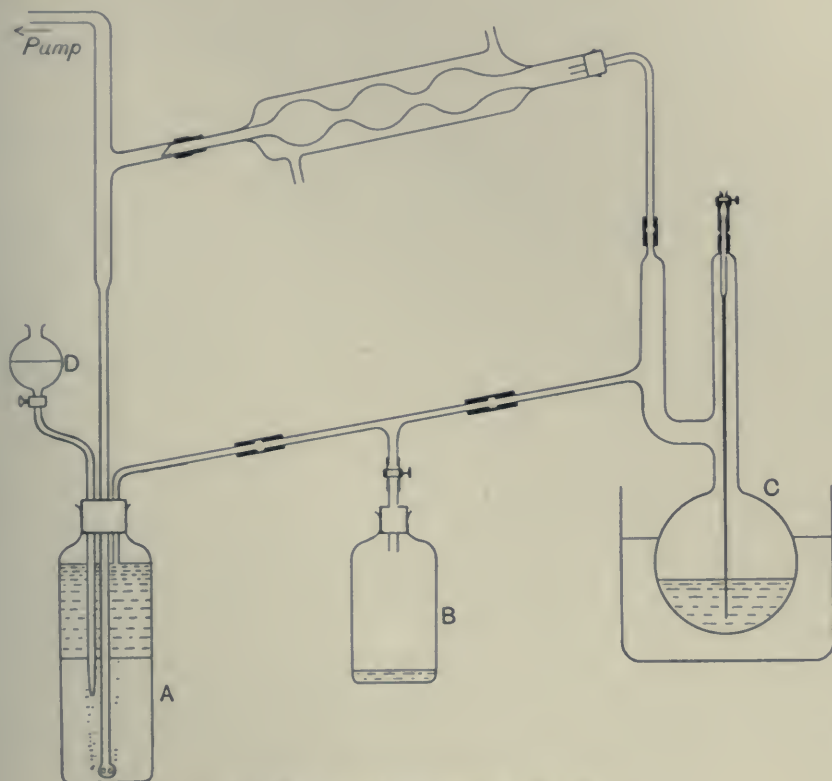
but he has been unable to find any evidence of the formation of urea from either ammonium carbonate or carbamate, and comes to the conclusion that the normal method of zymolysis of urea is by way of cyanic acid and ammonia, the former being rapidly hydrolysed by the solvent into more ammonia and carbon dioxide. Mack and Villars [1923, 1] have also isolated cyanates under similar conditions, but bring forward evidence [1923, 2] to show that when commercial urease is added to a very strong (10 *N*) solution of ammonium carbonate and carbamate at 55° (the optimum temperature for urease), small quantities of urea are produced and can be separated as the dixanthyl compound from the reaction mixture. They do not state whether any control experiments with heated inactivated urease were carried out, but find that a control without urease at the same temperature gives a very small precipitate with xanthhydrol.

There is no inherent chemical difficulty in the idea of the formation of urea from ammonium carbonate or carbamate. Lewis and Burrows [1912] showed that even at as low a temperature as 77°, on heating ammonium carbamate or urea in a sealed tube equilibrium was almost reached, after 95 days, at 1 % urea 99 % carbamate-carbonate. This same reaction at a higher temperature is described in a recent patent of the Badische Anilin und Soda Fabrik [1921] in which urea in 25 % yield is obtained by heating ammonium carbamate to 135°–140° under 15 atmospheres pressure.

But at ordinary temperatures and pressures this synthesis has not yet been observed, and the experiments about to be described indicate that the presence of urease is necessary if this synthesis is to take place at a measurable rate at room temperature.

The problem of showing that such a synthesis does take place in these circumstances is really that of separating urea in very minute quantity from a large excess of ammonium carbonate. The two substances are widely different in their chemical and physical properties, and separation may be achieved by taking advantage of the fact that whilst urea is soluble to the extent of 1.1 % in dry, and still more soluble in wet, butyl alcohol, ammonium carbonate is insoluble. Dakin's [1918, 1920] successful use of this partially miscible solvent in the continuous extraction of mono-amino acids from the products of protein hydrolysis led to my adopting it in an endeavour to effect this separation of urea. Since butyl alcohol boils at 118°, at which temperature small quantities of urea are rapidly decomposed, it was necessary to conduct the operation under reduced pressure, and a modification of the apparatus used by Dudley [1919] was employed. The temperature of the butyl alcohol was in this way kept at 60° during the course of the experiment, whilst a rapid stream of the solvent could be bubbled through the reaction mixture. This removed the urea from the reaction mixture almost as soon as it was formed, thus keeping its active concentration low and tending to force the reaction over towards further synthesis. The process of continuous removal from the sphere of reaction of substances synthesised by enzymes seems to

be a common biological phenomenon, and in this sense the method used in these experiments might be said to simulate what occurs in many living processes.



Apparatus used for continuous extraction.

A—reaction bottle.

B—bottle for excess butyl alcohol.

C—extraction flask containing butyl alcohol in bath at 60°.

D—dropping funnel containing urease.

Two preliminary experiments were made to find out whether butyl alcohol would extract urea quantitatively from its mixed solution with ammonium carbonate and carbamate:

(a) 1 g. of urea was dissolved in 100 cc. of a 10 % solution of dry ammonium carbonate, and extracted continuously for twelve hours with butyl alcohol. The residue in the extraction vessel was found to contain insufficient urea in 10 cc. to give a positive reaction with urease, which was easily given by 10 cc. of 0.05 % urea. Urea was shown to be present in the butyl alcohol in large quantities but was not estimated in this experiment. The extraction, then, is fairly complete.

(b) 0.001 g. of urea was dissolved in 100 cc. of a 20 % solution of ammonium carbonate, and extracted continuously for four periods of six hours

each, the separation and estimation of urea being conducted in the manner to be described later in this communication, using xanthhydrol.

					Wt urea extracted
First	six hour period	·0004 g.
Second	„ „	·00026 g.
Third	„ „	·00010 g.
Fourth	„ „	minute traces only

This gives a total quantity of about 75 % of the original urea extracted by this method from its very dilute solution in 18 hours.

By use of this apparatus, urea in small quantities has been isolated from the reaction mixture of urease + the equilibrium mixture of ammonium carbonate and carbamate which is obtained when strong ammonia solution is saturated with CO₂ gas at room temperatures and the resulting white crystalline precipitate is dissolved in distilled water. No urea whatever was obtained unless both active urease and this mixture were together present in the reaction bottle.

EXPERIMENTAL.

Ammonium carbonate. "Ammonium carbonate" was prepared by saturating portions of 250 cc. of 0·91 ammonia with dry CO₂ under a slight pressure. The precipitated salt was washed on a Buchner with a little cold distilled water, then with alcohol, and allowed to dry for two days in the air. On dissolving 20 g. of the dry solid in 100 cc. of water, some CO₂ was evolved and the resulting solution of p_H 8·2–8·4 was found to contain 84 % carbonate and 16 % carbamate, by Lewis and Burrows' [1912] method. During the extraction the p_H slowly increased, ending at 8·6–8·8, owing to loss of CO₂ from the solution.

Urease. Urease was prepared by two methods. In the earlier experiments it was obtained by extracting freshly ground soy bean meal with ten times its weight of 0·3 % KH₂PO₄ and allowing to stand for ten minutes. The mixture was then filtered through paper, allowed to stand for at least half-an-hour, and the activity of the urease was tested using a standard buffered solution of urea. It was then filtered into the dropping funnel shown on the left in the diagram, and covered by a layer of butyl alcohol to prevent any slight risk of bacterial contamination. Usually 20 cc. of the enzyme solution was used for a day's (8 hours) extraction, the liquid being added in 1 cc. portions at intervals. This procedure was adopted owing to the fact that butyl alcohol plus ammonium carbonate slowly destroy the enzyme.

Onodera [1915] showed that whilst in small concentrations the monohydric alcohols up to amyl alcohol increased the activity of soy bean urease (this increase being connected with the diminution in surface tension at the interface between the colloidal particles of the urease and the continuous phase), in higher concentrations the action was inhibitory. In a series of preliminary

trials the inhibitory effect of large concentrations of butyl alcohol was confirmed. Some idea of the relative activity of the enzyme before and after the experiment was obtained by noting the times taken by strictly comparable dilutions of the fresh enzyme and of the enzyme from the reaction mixture to change the p_H of a standard buffered solution of urea from 7.0 to 8.0. After a day's extraction the activity gauged in this way was diminished to one-fifth or less. Some of this effect was due to the ammonium carbonate, which on standing with urease for eight hours was found to diminish its activity, as previously observed by Barendrecht and others.

In the later experiments urease was prepared by Van Slyke and Cullen's method [1914] by two precipitations with ten volumes of acetone. The criticism that the urea found might have been present in some protected form in the cruder preparation could not arise in the case of this purified urease. 0.5 g. of the solid, dissolved in 20 cc. of distilled water, and filtered through glass wool was used for each day's (8 hours) extraction.

Methods of detection and analysis of urea. At the end of the period of continuous extraction, the stop-cock leading to the bottle *B* was opened and the butyl alcohol remaining in the extraction flask was distilled into this bottle until the flask was almost dry. The pressure was then released and the contents of the flask washed out with three washings of absolute alcohol into a small evaporating basin. The alcohol was evaporated on a water-bath and the dry residue washed three times with boiling chloroform. This removed most of the colouring matter which had come over in the butyl alcohol and left behind an almost colourless residue which was then extracted three times with 10 cc. of boiling acetone. The filtered acetone solution was evaporated to dryness, and the very small quantity of crystalline residue contained any urea which had been present in the extraction flask at the close of the extraction period. In later experiments it was found that the chloroform always extracted, together with the colouring matter, a trace of urea which could be detected by xanthhydrol precipitation but which was insufficient to weigh.

Two methods were used for the detection and estimation of the urea in the acetone extract.

1. The well-known method, by which the p_H of an indicator solution is changed to the alkaline side when urease of a known p_H and urea solution at the same p_H are added together and warmed to 37° for a short period, was extended to work with very small quantities of urea as follows:

A phosphate buffer mixture ($M/15$ KH_2PO_4 and $M/15$ $KNaHPO_4$) was made of $p_H = 7.0$, and 0.1 cc. of this was added to each of a series of tubes containing 5 cc. of dilute solutions of urea of different, known strengths with 0.5 cc. of 0.01 % phenol red.

Urease was prepared by diluting 5 cc. of the soya bean extract with an equal quantity of water, and adjusting the p_H to 7.0 with a few drops of $N/50$ NaOH. Of this enzyme solution 0.2 cc. was added to each of six tubes containing urea, the p_H immediately noted, and noted again at intervals.

Tube	Amount urea in mgm.	p_{H} at start	p_{H} after 15 mins. at 14°	p_{H} after raising to 37° for 15 mins.	p_{H} after 90 mins. at 37°
1	1.25	7.0	>8.4	>8.4	>8.4
2	0.25	7.0	7.8	7.9	7.9
3	0.10	7.0	7.35	7.4	7.4
4	0.05	7.0	7.1	7.1	7.15
5	0.025	7.0	7.0	7.1	7.1
6	0.012	7.0	7.0	7.0	7.0
7	1.25	Control with boiled enzyme no change of p_{H}			

This method is therefore capable of detecting 0.05 mgm. of urea in 5 cc. of liquid, 1 in 100,000, and in quantities somewhat greater than this the change of p_{H} gives a rough quantitative idea of the amount of urea present. Armstrong has shown how very specific the action of urease is, and this definite change of p_{H} after the addition of urease is convincing evidence of the presence of urea.

The advantage of this method was that it was possible to follow it by an estimation of the amount of ammonia produced from the synthesised urea by the enzyme, using a micro-Folin apparatus. Potassium carbonate was added to the reaction mixture and the ammonia liberated was aspirated into $N/100 \text{ H}_2\text{SO}_4$ and titrated with $N/100 \text{ CO}_2$ -free soda in a CO_2 -free atmosphere using methyl red as the indicator. The serious drawback was that half of the solution of urea had to be reserved for a blank determination with heated enzyme. It was always necessary to do this, as the blank gave from one-third to one-sixth of the total amount of ammonia produced by the active enzyme.

2. Xanthhydrol was used in the later experiments for the detection and estimation of urea using Nicloux and Welter's [1921] gravimetric modification of Fosse's well known method.

The acetone solution of urea obtained from the contents of the extraction flask in the usual way was evaporated to dryness and the minute crystalline residue dissolved in 2 cc. of water and filtered. The filter paper was washed with a little water, and to the 3 cc. of urea solution thus obtained were added 5 cc. of glacial acetic acid and 0.5 cc. of a 10 % solution of xanthhydrol in methyl alcohol. The liquid was shaken and allowed to stand at room temperature for ten minutes, when a crystalline precipitate could be observed uniformly throughout the tube. This began to separate, and after two to three hours the liquid was filtered off through a Neubauer micro-crucible and the dixanthyl urea washed and dried as described by Nicloux and Welter. The weight of urea is exactly one-seventh of the weight of the dixanthyl urea.

Results. These are tabulated below. About two-thirds of the experiments which have been done are given. Those omitted from considerations of space are duplicates completely confirmatory of the results shown.

The quantities of urea obtained are small, but quite recognisable and capable of estimation. In the later experiments as the technique improved the yields of urea became somewhat greater. Endeavours were made to obtain

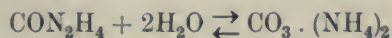
Enzyme	Substrate	Time of extraction in hours	Method of testing for urea	Method of estimation	Amount urea found in mgm.
Soya bean extract	$(\text{NH}_4)_2\text{CO}_3$	6	Urease	Urease	>0.1
"	Distilled water only	6	"	"	Nil
"	$(\text{NH}_4)_2\text{SO}_4^1$	6	"	"	"
"	$(\text{NH}_4)_2\text{SO}_4$ at $p_{\text{H}}=8.6$	6	"	"	"
"	Distilled water	16	"	"	"
"	$(\text{NH}_4)_2\text{CO}_3$	18	"	"	0.3*
"	"	18	"	Micro-Folin	0.2
None	"	18	"	Urease	Nil
"	"	20	Xanthhydrol	Xanthhydrol	"
Soya bean extract boiled	"	15	Urease	Urease	"
Soya bean	"	45	"	Micro-Folin	>0.6
Van Slyke urease	"	26	Xanthhydrol	Xanthhydrol	0.37
"	"	16	Urease	Micro-Folin	0.24
None	"	20	Xanthhydrol	Xanthhydrol	Nil
Van Slyke urease ²	"	20	"	"	0.30
"	"	12	"	"	0.35

¹ Of same concentration in NH_4 ions as the ammonium carbonate used in the same series of experiments.

² A particularly active preparation.

* Reaction mixture in bottle kept at 37° during last eight hours of this experiment.

larger quantities of urea by conducting the operation in strong cane sugar solutions, and thus forcing the simple reaction



over towards the side of synthesis by diminishing the amount of available water, but the results were not encouraging:

Enzyme	Substrate	Time of extraction in hours	Method of testing and estimation	Amount of urea found in mgm.
Soya bean extract	$(\text{NH}_4)_2\text{CO}_3$	8	Urease	0.1
"	$(\text{NH}_4)_2\text{CO}_3$ in 25 % cane sugar	14	"	0.1
"	$(\text{NH}_4)_2\text{CO}_3$ in 50 % cane sugar	20	"	Traces only

No further attempts have been made, up to the present, to increase the yield.

The actual change in colour of the indicator, on hydrolysis of half of the urea solution (the other half being kept as control) by means of the same enzyme that had synthesised it, was most striking, particularly with the larger quantities of urea. In the "synthesised" urea solution the rate of colour change was the same as in a solution of urea of the same approximate strength made up for comparison.

In two experiments not given above the synthesised urea was crystallised from a very small quantity of acetone (by evaporation) and compared microscopically with a similar quantity of ordinary urea similarly treated. The resemblance of crystalline form was evident. A small portion of the synthesised urea placed on the outside of a test-tube containing boiling butyl alcohol (118°) did not melt, but when put onto the outside of a test tube in which sulphuric acid was being heated melted below 126° . (Urea melts at 132° .) There was insufficient for a m.p. determination in the ordinary way. The urea

still contained a little pigment. On heating in a small dry tube, ammonia was given off, but as this reaction meant the disappearance of the yield of 18 hours' extraction it was not repeated.

DISCUSSION.

It has been established by these experiments that urea may be extracted from a solution containing *active* urease and ammonium carbonate-carbamate mixture, but not under conditions in which the enzyme is destroyed, or in which either the ammonium carbonate or the active enzyme is absent.

Control experiments as indicated above have shown that the urea does not come from

- (a) ammonium carbonate + carbamate alone;
- (b) the enzyme solution alone;
- (c) the enzyme in presence of another ammonium salt;
- (d) the inactivated enzyme in presence of ammonium carbonate and carbamate.

The control experiments also negative the possibility that the urea found is derived from the oxidation of the butyl alcohol in presence of ammonia to cyanic acid and the transformation of this substance into urea [cf. Fosse and Laude, 1921]. A solution of ammonium sulphate made alkaline with ammonia in presence of active urease was found to give no trace whatever of urea under the same conditions of experiment in which ammonium carbonate gave easily recognisable quantities.

A fair summary of the situation with regard to the various reactions which may theoretically take place during the hydrolysis and synthesis of urea is given by Mack and Villars [1923, 1], and the experiments described above fall most easily into line with the conclusion of these authors that the reaction $\text{urea} \rightleftharpoons \text{ammonium carbamate}$ is the one catalysed by urease and not the reaction $\text{urea} \rightleftharpoons \text{ammonium cyanate}$, or $\text{urea} \rightleftharpoons \text{cyanic acid} + \text{ammonia}$. In the latter case it would be necessary also for the urease to catalyse the reaction $\text{cyanic acid} + \text{ammonia} \rightleftharpoons \text{ammonium carbonate}$ which, as has been known for some time [Walker and Hambley, 1895], is a very slow reaction in alkaline solution even at 69°, and at equilibrium only gives about 4 % of ammonium carbonate. The great rapidity and the completeness with which urea is hydrolysed by urease would therefore seem, in themselves, to put this explanation out of court.

Further experiments are being carried out in an endeavour to throw more light on the mechanism of the action of urease.

SUMMARY.

Urea is produced in very small quantities during the action of urease on a mixture of ammonium carbonate and carbamate in strong solution in water, at room temperatures, under conditions which preclude the formation of urea by any other means than enzymic synthesis. The action of urease is, therefore, reversible.

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XXXIII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

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(Received March 20th, 1923.)

AMONG the esters of phosphoric acid possessing biochemical interest three are known in which the phosphoric acid group is believed to be combined with a hexose molecule.

Hexosediphosphoric acid was discovered by Harden and Young [1905] and by Ivanov [1905] among the products of the fermentation of glucose, fructose or mannose by yeast juice in presence of phosphates, and was shown by Young [1909, 1911] to possess the constitution $C_6H_{10}O_4(PO_4H_2)_2$ and to yield fructose on hydrolysis. One of the two phosphoric acid molecules is very easily removed by boiling the ester with weak acids and in this way Neuberg [1918] obtained a hexosemonophosphoric acid $C_6H_{11}O_5(PO_4H_2)$ which like the diphosphoric ester is very slightly dextrorotatory and yields a laevo-rotatory sugar on further hydrolysis.

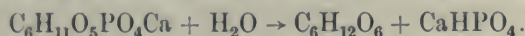
The presence of a hexosemonophosphoric ester was observed by Harden and Robison [1914] among the products of fermentation of sugar by yeast juice, and the isolation and investigation of this ester has been described by Robison [1922] in a recent number of this Journal. It is entirely different from the compound prepared by Neuberg, being strongly dextrorotatory and yielding a dextrorotatory sugar on hydrolysis. The phosphoric acid group in this ester is moreover much more slowly removed by boiling with acids than either of the groups in hexosediphosphoric acid.

The barium and calcium salts of the diphosphoric ester are sparingly soluble in cold and still less soluble in hot water. Those of the monophosphoric esters are on the contrary very easily soluble in water.

In animal physiology hexosediphosphoric acid has acquired great interest and significance through the researches of Embden and his co-workers who have brought forward convincing evidence [Embden and Lacquer, 1921] that this or some closely related phosphoric ester is present in striped muscle and is identical with the "lactacidogen."

During my investigation of the hexosemonophosphoric acid isolated from the products of fermentation, the hydrolysis of the ester by enzymes was studied. In some experiments in which the readily soluble calcium and barium

salts were used as substrate, the progress of the hydrolysis was shown by the formation of a precipitate of sparingly soluble calcium or barium phosphates,



The formation of this precipitate suggested to me the query whether some such reaction might conceivably be concerned in the deposition of calcium phosphate during the formation of bone in the animal body. In the first instance I sought for the presence of an enzyme capable of effecting such hydrolysis in the bones of growing animals.

Experiment 1. Aqueous extract of bone of rabbit. The femur taken from a young rabbit immediately after death was pounded with water saturated with toluene and after two hours the extract was filtered. 0.1 cc. of this extract, equivalent to 0.02 g. fresh bone was incubated at 37° with 2 cc. of a 1 % solution of barium hexosemonophosphate + 0.05 cc. toluene. Controls containing (1) 0.1 cc. extract in 2 cc. water, (2) 2 cc. barium hexosemonophosphate solution without extract, were incubated at the same time. Within 18 hours a precipitate of barium phosphate had formed in the solution containing the phosphoric ester + bone extract but not in either of the controls. After 60 hours at 37° the free phosphate in all three tubes was estimated, the results indicating that 35 % of the total phosphoric acid had been hydrolysed by the bone extract.

The free phosphoric acid was estimated by precipitation with ammonium molybdate as in Neumann's method, omitting the previous ashing with nitric and sulphuric acid. The exact method employed was as follows:—A solution containing 5 cc. concentrated sulphuric acid, 25 cc. of a 50 % solution of ammonium nitrate, and 60 cc. water is heated to 75°–80°. The phosphate solution to be estimated is then added, followed immediately by 20 cc. of a 10 % solution of ammonium molybdate. If the volume of the phosphate solution is much greater than 10 cc. the amount of water is correspondingly reduced. The temperature of the solution before the addition of the ammonium molybdate should be 70°–75°. The flask is vigorously shaken and allowed to stand for $\frac{1}{4}$ – $\frac{1}{2}$ hr. The yellow precipitate is then filtered off in the special funnel described by Plimmer and Bayliss [1906], washed with ice water and estimated in the usual way with standard NaOH solution, 0.1N solution being employed when the amount of free phosphoric acid corresponds with less than 2 mg. P. Carbon dioxide must be expelled by adding a slight excess of acid and boiling for a few minutes before the final titration.

With this method very varying quantities of phosphorus can be estimated with an accuracy of about 0.01 mg. (using 0.1N solution) or indeed, as Iversen [1920, 1, 2, 3] has shown, a still greater degree of accuracy may be obtained if special precautions are taken.

It cannot, of course, be taken for granted that no organic ester of phosphoric acid will be hydrolysed under the above conditions, but actual tests showed that with solutions of hexosemonophosphoric acid, glycerophosphoric

acid and even hexosediphosphoric acid, the amount of hydrolysis is negligible provided the temperature is not allowed to rise or the time prolonged unduly.

The total phosphorus was estimated in all cases by Neumann's method.

Experiment 2. Bones of a young rat (age 16 days, weight 28 g.). The epiphyseal cartilage was removed from both ends of each bone. The cartilage and shaft from one femur, tibia and humerus were each separately incubated 22 hours at 37° with 1 cc. of a solution of potassium hexosemonophosphate (sol. A) + 0.05 cc. chloroform. The cartilage and shaft of the second femur and tibia and the shaft of the humerus were incubated with 1 cc. water + 0.05 cc. chloroform. The cartilage of the second humerus was heated to 100° for a few minutes and then incubated with solution A. The weight of bone or cartilage in each tube was not accurately determined but was about 0.05 g.

Experiment 3. Bones of a rachitic rat. These were treated as described above—cartilage and shaft being separately tested.

The results of experiments 2 and 3 are shown in Table I.

Table I.

Experiment	Total P mg. P in 1 cc. sol. A	Free PO ₄ after 22 hours at 37° (mg. P)				Percentage of combined P hydrolysed by tissue
		1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	
2. Normal rat:						
Femur shaft	3.53	0.10	0.00	1.76	1.66	48
" cartilage	"	"	0.00	1.16	1.06	31
Tibia shaft	"	"	0.00	1.86	1.76	51
" cartilage	"	"	0.00	1.35	1.25	36
Humerus shaft	"	"	0.00	1.27	1.17	34
" cartilage	"	"	—	1.33	1.23	36
Humerus cartilage previously heated to 100°	"	"	—	0.09	-0.01	0
3. Rachitic rat:						
Humerus shaft	3.72	0.10	0.03	2.60	2.47	68
" cartilage	"	"	0.07	2.95	2.78	77

These results clearly demonstrate the presence of an enzyme which can effect the hydrolysis of hexosemonophosphoric acid in the bones and ossifying cartilage of young rats and certainly not less in those of rachitic than those of normal animals. The next step was to discover whether the enzyme is present in other tissues also. For this purpose a young rat from the same litter as those used in the previous experiments was taken.

Experiment 4. Tissues of young rat (age 30 days, weight 48 g.). Weighed quantities (0.05 g. \pm 5 %) of the various tissues were removed with all precautions against bacterial contamination and were incubated with 1 cc. of potassium hexosemonophosphate solution + 0.05 cc. chloroform (series A) or with 1 cc. of water + 0.05 cc. chloroform (series B). After 46 hours at 37° the contents of each tube were diluted with 5 cc. distilled water and filtered through a small Buchner funnel, the tube and funnel being rinsed out three times with 5 cc. water. Free phosphate was estimated in the filtrate and washings. The results are given in Table II. In the case of the spleen and

trachea the weight of tissue available did not permit of 0.05 g. being taken and the amount of phosphoric acid formed by hydrolysis has therefore been multiplied by the appropriate factor in order that the figures (enclosed in brackets) may be compared with those for the other tissues.

Table II.

Tissue	Weight g.	Total P mg. in 1 cc. sol. A	Free PO_4 after 46 hours at 37° (mg. P)				Percentage of combined P hydrolysed by tissue
			1 cc. sol. A	Tissue + water	Tissue + sol. A	Difference	
Femur cartilage (epiphysis)	·05	3.72	0.16	0.10	3.47	3.21	90
Rib cartilage	·05	"	"	0.08	0.30	0.06	2
Trachea	·02	"	"	0.04	0.30	0.10 (0.25)	3 (7)
Liver	·05	"	"	0.06	0.62	0.40	11
Spleen	·04	"	"	0.06	0.45	0.23 (0.29)	7 (8)
Thymus	·05	"	"	0.28	0.50	0.06	2
Heart muscle	·05	"	"	0.07	0.55	0.32	9
Pancreas	·05	"	"	0.21	0.48	0.11	3
Kidney	·05	"	"	0.15	2.59	2.28	64

The figures in the last column give the percentage of combined phosphoric acid set free by the tissue enzyme and point to an almost unique position for ossifying as distinct from non-ossifying cartilage in respect of this enzyme. Of the other tissues, though slight hydrolysis was produced in all cases, only the kidney approached the femur epiphysis in enzymic power. The trachea and rib cartilage are particularly interesting as examples of non-ossifying cartilage, both showing a very low hydrolytic power. In this experiment the ribs themselves were not examined nor the costochondral junctions, the cartilage being specially taken at some distance from the latter, but in a later experiment the costochondral junctions were found to contain the enzyme in a high degree and to be entirely comparable with cartilage of other bones in this respect.

In all the above experiments salts of hexosemonophosphoric acid were employed as substrate. In the next the action of various tissues on salts of hexosemonophosphoric, hexosediphosphoric and of glycerophosphoric acid was compared.

Experiment 5. Tissues of rabbit (age under 4 weeks). The tissues were macerated with twice their weight of water containing 2 % of chloroform until a fairly uniform mixture was obtained. 0.3 cc. of this maceration mixture, equivalent to 0.1 g. of fresh tissue was placed in each of the following solutions:

Sol. 0. 2 cc. water + 0.1 cc. chloroform

Sol. 1. " potassium hexosemonophosphate sol. (combined P = 5.47 mg.) + 0.1 cc. chloroform

Sol. 2. " hexosediphosphate sol. (combined P = 4.00 mg.) + 0.1 cc. "

Sol. 3. " glycerophosphate sol. (combined P = 6.58 mg.) + 0.1 cc. "

All solutions were incubated for 40 hours at 37° and were then filtered and the free phosphate estimated as described above. 2 cc. of sols. 1, 2 and 3 were also incubated with 0.1 cc. chloroform but without the addition of any tissue, as controls. The traces of free phosphate present in these solutions were not increased by the incubation, indicating that no appreciable hydrolysis takes

place in the absence of tissue under these conditions. To avoid multiplying the number of columns these small amounts have been subtracted from the corresponding totals and are not shown in the table (Table III). The amount of free phosphate arising from autolysis of the tissues is given in the first column, and this has also been subtracted from the totals in order to arrive at the amount of phosphoric ester hydrolysed by the tissue, as shown in the remaining columns.

Table III.

Tissue	Free phosphate after 40 hours' incubation at 37° (mg. P)				Percentage of phosphoric ester hydrolysed by tissue		
	Tissue + sol. 0 (autolysis)	Tissue + sol. 1 less P in controls	Tissue + sol. 2 less P in controls	Tissue + sol. 3 less P in controls	Sol. 1 potassium hexosemono-phosphate	Sol. 2 potassium hexosediphosphate	Sol. 3 potassium glycerophosphate
Femur cartilage (epiphysis)	0.13	5.19	3.56	3.64	95	89	56
Rib cartilage	0.01	0.45	2.14	0.39	8	54	6
Trachea cartilage	0.11	—	1.31	—	—	33	—
Blood	0.03	0.02	0.01	0.53	0	0	8
Liver	0.15	1.19	2.62	1.54	22	66	24
Spleen	0.22	1.18	1.98	0.89	22	50	14
Pancreas	0.20	0.31	0.92	0.37	6	23	6
Muscle	0.17	0.08	1.98	0.15	1	50	2
Kidney	0.16	2.22	3.17	1.33	41	79	20

The figures for sol. 1 (potassium hexosemonophosphate) follow in general the same order as those in Table II, and show the same striking superiority of the epiphyseal cartilage over other tissues with respect to this enzyme. The hydrolysis of the ester by the femur cartilage is almost complete while the kidney, which is again second, only hydrolysed 41 %. Liver and spleen were relatively rather more active than in the previous experiment while with muscle and blood practically no hydrolysis was shown. A somewhat similar order is found in the figures for sol. 3 (potassium glycerophosphate) though the degree of hydrolysis is usually less than with hexosemonophosphate¹. Whether the somewhat pronounced variations between the two sets of figures, *e.g.* in those for liver and blood, indicate that a different enzyme is involved, can only be decided by further experiments. The action of tissue enzymes on glycerophosphates has been investigated by Grosser and Husler [1912] who found that kidney and intestine effected complete hydrolysis in 24 hrs. at 37°, liver only 16 %, while spleen, blood and muscle were inactive. Plimmer [1913] obtained similar results with kidney and intestine, but found extracts of liver and pancreas to be inactive. In these as in my own experiments the glycerophosphate employed was the commercial preparation and probably contained both α and β varieties. The results obtained by these investigators and my own (including experiments not recorded in this paper) point to a variable but usually very low hydrolytic power for liver and liver extracts with respect to both glycerophosphoric acid and hexosemonophosphoric acid. The figures for sol. 2 (potassium hexosediphosphate) in Table III

¹ The difference in the rate of hydrolysis of potassium hexosemonophosphate and potassium glycerophosphate has not been confirmed in later experiments and may have been due to slight differences in the p_H of the two solutions. (Note added April 13th.)

differ considerably from those for the other esters and appear at first sight to show no striking variations among themselves. On closer examination however, one interesting fact emerges, that nearly all the tissues examined possess an enzyme capable of hydrolysing up to 50 % of the combined phosphorus—i.e. one of the two phosphoric acid groups. Thus muscle and rib cartilage, which are almost without action on hexosemonophosphoric acid and glycerophosphoric acid both hydrolyse approximately 50 % of the phosphorus in the diphosphoric ester. Only blood is entirely inactive. The hydrolysis of the second phosphoric acid group appears then to follow approximately the same order as that for hexosemonophosphoric acid. Obviously two different enzymes are here involved. The very different hydrolytic power of muscle with regard to the two phosphoric acid groups is significant in view of the close relationship between hexosediphosphoric acid and lactacidogen.

Tomita [1922, 1 and 2] has also investigated the action of animal tissues on the synthetic sucrose monophosphate, and on Neuberg's hexosemonophosphate and has found them to possess hydrolytic power diminishing in the following order: kidney, liver, spleen, pancreas, muscle.

DISCUSSION OF RESULTS.

It has thus been shown that the ossifying cartilage of young rats and rabbits contains a very active enzyme which rapidly hydrolyses hexosemonophosphoric ester and also glycerophosphoric ester. The kidney also contains this enzyme but its hydrolytic power is considerably less, for equal weights of tissue, than that of ossifying cartilage. Other tissues examined, including non-ossifying cartilage from ribs and trachea, contain the enzyme in very much smaller degree or not at all.

The possible significance of these facts in processes of ossification may now be considered. The cause of the deposition of calcium phosphate in the growing bone and its non-deposition elsewhere has not been explained. It has been calculated [Adler, 1908] that an aqueous solution containing the same amounts of inorganic salts as are found in serum would deposit a mixture of calcium phosphate and carbonate. The increased solubility of these salts in presence of serum proteins has been given [Pauli and Samec, 1909] as an explanation of the non-formation of such a precipitate in blood while specific adsorption of ions by the cartilage has been suggested [Pfaundler, 1904] to explain the deposition of calcium salts in bone. It has been shown by many investigators that blood contains in addition to lipid phosphorus and inorganic phosphate a certain amount of combined phosphoric acid which is readily hydrolysed by boiling with acids. Thus Greenwald [1916] found a small amount of such combined phosphoric acid (less than 1 mg. P per 100 cc.) in serum. Bloor [1918] found similar amounts of organic phosphate (10 % of the total phosphates) in plasma of normal men and women but much greater amounts (60–80 % of the total phosphates) in the corpuscles. Jones and Nye [1921] have obtained similar figures for corpuscles from the blood of children of both sexes but

think that the unknown phosphoric acid in plasma is negligible in amount. In a later paper McKellips, De Young, and Bloor [1921] give analyses of blood of very young infants and find that the amount of this unknown phosphoric acid in the plasma is much higher (average 2.2 mg. P per 100 cc.) than for adults.

From a consideration of the work of these investigators it appears very probable that some ester of phosphoric acid which is soluble in acids but insoluble in ether and which is not precipitable by magnesia mixture or by ammonium molybdate is present in small amounts in plasma and in relatively large amounts in the corpuscles. If this ester is hydrolysable by the enzymes of ossifying cartilage the result would be an increase of HPO_4'' ions in the cartilage. If a condition of equilibrium exists between solid calcium phosphate on the one hand and a solution containing un-ionised calcium phosphate, and calcium and phosphate ions on the other, any increase in the concentration of either of these ions will result in further deposition of the solid calcium phosphate.

I would therefore suggest that the hydrolysis of some phosphoric ester, the calcium salts of which are readily soluble in water, by the enzyme in the ossifying cartilage, with production of free phosphoric acid may possibly be a factor in the process of bone formation. Whether this ester is a hexosemonophosphoric acid can only be decided by its isolation and this I am attempting to carry out. In conjunction with Mr H. D. Kay, experiments have already been performed which indicate with considerable probability that the "acid soluble" organic phosphate of blood is hydrolysed by the ossifying cartilage of young animals. The problem is also being attacked in another way with promising results. A preliminary experiment may be briefly described.

Bones from a severely rachitic rat were split lengthwise and one-half of each immersed in a solution of calcium hexosemonophosphate—the other half being immersed in normal saline. Both were incubated at 37° for two days in presence of chloroform and were then removed, washed with distilled water and fixed in formalin. Sections of these bones were made and stained with silver nitrate. The half which had been incubated with saline showed the customary greatly enlarged zone of provisional calcification practically devoid of phosphate while the half bone which had been incubated with the calcium hexosemonophosphate solution showed an intense black stain in the matrix of the cartilage cells when treated with silver nitrate. This may conceivably have been due merely to adsorption of the ester, but it was noted that the deposit occurred only in and throughout the depth of the zone of provisional calcification and not in the proliferating cartilage as might have been expected if adsorption were the sole cause. Further experiments must decide this point. For the histological investigations, as well as for the supply of the rachitic bones and for advice on this aspect of the work I am very greatly indebted to the kindness of Dr H. Goldblatt to whom I would here express my thanks.

SUMMARY.

An enzyme is present in the ossifying cartilage of young rats and rabbits, which rapidly hydrolyses hexosemonophosphoric acid, yielding free phosphoric acid.

In respect of this enzyme the kidney is considerably less active (about 50 %) than an equal weight of epiphyseal cartilage. Other tissues contain the enzyme in a very much lower degree, muscle and blood being almost inactive.

Non-ossifying cartilage shows less than one-tenth of the hydrolytic power of ossifying cartilage.

The same tissues in approximately the same order also hydrolyse glycerophosphoric acid.

One of the two phosphoric acid groups of hexosediphosphoric acid is very readily hydrolysed by almost all tissues (including muscle and non-ossifying cartilage) except blood.

The possible significance of this enzyme in the process of ossification in the animal body is discussed and certain preliminary experiments in various directions are briefly described, by the further prosecution of which it is hoped to obtain more information on this subject.

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XXXIV. A STUDY OF RATS ON A NORMAL DIET IRRADIATED DAILY BY THE MERCURY VAPOUR QUARTZ LAMP OR KEPT IN DARKNESS.

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HISTORICAL.

IN the course of a study on the absence of rickets during confinement on a good diet, Mellanby [1921] kept four puppies in small kennels in the dark for a period of thirteen weeks. Their diets consisted of:

- (a) bread, milk and meat;
- (b) bread, milk, meat and bone;
- (c) bread, milk, meat and bone that had been autoclaved at 120° for 15 minutes.

The puppies were ten weeks old at the beginning of the experiment and were killed at the age of 26 weeks. Histologically, no sign of rickets was found. Chemical analysis of the shafts of the femurs gave the following percentages of CaO in the dry bone:

Puppy No. 25	42.92
„ 26	38.13
„ 27	37.46
„ 28	30.00

These values Mellanby considers to be high. It is evident from the foregoing facts that the calcium content of the bones was unaffected by 13 weeks of confinement in the dark.

Raczynsky [1912] investigated the effect of sunlight and darkness on puppies during the period of lactation. He took two puppies from the same litter, born in May, kept one in total darkness from birth and the other in sunlight, both suckled by the same mother. After six weeks they were killed

and the bodies chemically analysed. The result is given in the following table:

	Per 100 g. of body weight	
	Puppy in sunlight g.	Puppy in darkness g.
CaO	1.58	0.98
P ₂ O ₅	1.19	0.86
MgO	0.05	0.04
Cl	0.16	0.35
Fe	0.02	0.02

From the fact that the calcium and phosphorus content of the puppy kept in the dark was lower than that of the one kept in sunlight, he surmised that the former had rickets. The diet of the mother is not known, therefore it cannot safely be assumed that the puppies received an optimal amount of calcium, phosphorus and anti-rachitic organic factor. Comparing the results with those about to be recorded, one ventures to suggest that the mother's diet probably was not complete.

EXPERIMENTAL.

Radiation. Since sunlight was not available, the radiations from a mercury vapour quartz lamp were used as a substitute. The lamp employed is a Pistolette, manufactured by the Hewitt Electric Co. The shortest wave length of its spectrum is $230\mu\mu$ and a daily twenty minute exposure to the rays from this lamp has been found to prevent or cure rickets in rats on diet No. 3143 of McCollum and his collaborators [1921], and on other rickets-producing diets.

Two litters of rats were divided into two groups with an equal number of the same sex in each. One group was kept in total darkness from the time the rats were weaned, while the other was kept in a room with a large double window consisting of plate glass about half-an-inch thick, through which daylight had to filter to enter the room. But the latter group was exposed for 20 minutes daily at a distance of 60 cm. to the radiations from the mercury vapour quartz lamp.

Diets. In the case of litter XXI, both groups received the following diet:

Diet N 1	Inactivated commercial "casein"	...	20.0 g.
	Starch (wheat)	50.0
	Cotton-seed oil (hardened)	10.0
	Cod-liver oil	5.0
	Salt mixture (McCollum No. 185)	5.0
	Calcium carbonate	3.1
	Marinite	5.0
	Lemon juice	5.0 cc.
	Distilled water	46.9 cc.

In the above diet the percentage of calcium in the paste was 1.07, of phosphorus 0.53 and of cod-liver oil 3.66, all optimal amounts for these substances.

The two groups of litter XXII were subdivided so that half the rats in each group (darkness and light) received the same diet as litter XXI and the other half a similar diet in which butter was substituted for both the cod-liver and cotton-seed oils. This diet consisted of:

<i>Diet N 2</i>	Inactivated commercial "casein"	...	20.0 g.
	Starch (wheat)	50.0
	Fresh butter	15.0
	Salt mixture (No. 185)	5.0
	Calcium carbonate	3.1
	Marmite	5.0
	Lemon juice	5.0 cc.
	Distilled water	46.9 cc.

The rats of litter XXI were 23 days and those of litter XXII 28 days old at the beginning of the experiment. At the end of eight weeks both groups were killed. That experimental period was chosen as it was the one used most frequently in other investigations and because the rats were then about twelve weeks old and therefore nearly full-grown.

Table I.

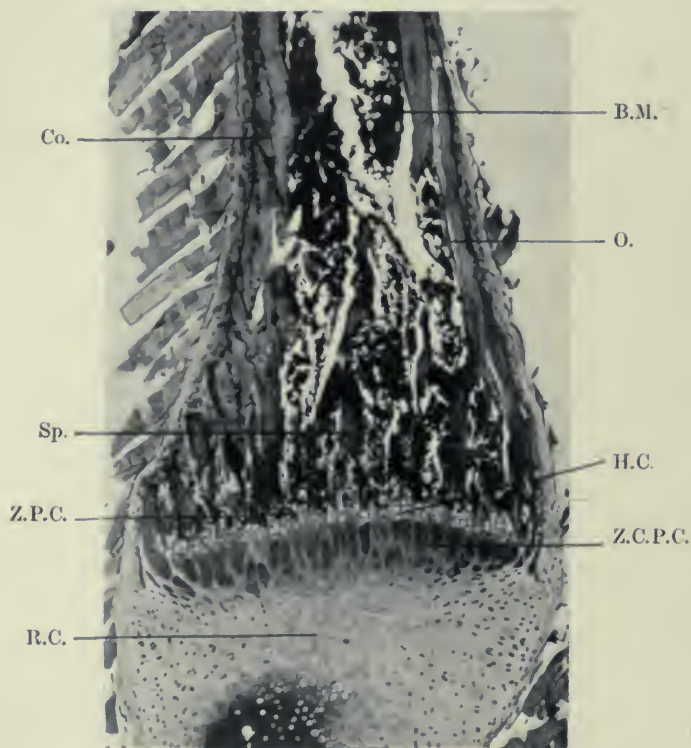
No. of rat	Sex	No. of litter	Diet	Age (in days)		Days on diet	Average amount of food eaten daily	Weight grams		Gain grams	In bones		
				Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry
G. 409	♀	XXI	N 1	23	79	56	12.6	28	101	73	40.95	12.51	21.21
G. 410	♂	XXI	N 1	23	79	56	17.2	34	172	138	40.10	12.67	21.15
G. 411	♂	XXI	N 1	23	79	56	16.0	38	161	123	40.88	12.51	21.13
Averages											40.64	12.56	21.16
G. 412	♀	XXI	N 1	23	79	56	12.2	33	113	80	38.25	12.80	20.73
G. 413	♂	XXI	N 1	23	79	56	15.0	33	139	106	38.12	12.72	20.55
G. 414	♂	XXI	N 1	23	79	56	17.5	38	160	122	42.28	12.22	21.15
Averages											39.55	12.58	20.81
G. 424	♀	XXII	N 1	28	84	56	14.5	31	123	92	41.46	13.05	22.28
G. 425	♂	XXII	N 1	28	84	56	21.1	36	198	162	40.00	13.61	22.67
Averages											40.73	13.33	22.47
G. 426	♀	XXII	N 1	28	84	56	14.6	27	131	104	38.95	13.69	22.68
G. 427	♂	XXII	N 1	28	84	56	17.7	34	191	157	39.85	13.28	22.07
Averages											39.40	13.48	22.37
G. 428	♀	XXII	N 2	28	84	56	22.0	32	142	110	37.14	14.19	22.58
G. 429	♂	XXII	N 2	28	84	56	19.8	31	178	147	39.65	13.84	22.95
Averages											38.38	14.01	22.76
G. 430	♀	XXII	N 2	28	84	56	16.5	29	136	107	35.85	14.56	22.70
G. 431	♂	XXII	N 2	28	84	56	18.5	33	193	160	38.83	14.28	23.34
Averages											37.34	14.42	23.02

NOTE. The histological diagnosis in each rat is normal.

Table II.

All rats on normal diets	Average % H ₂ O	Average % Ca (wet bones)	Average % Ca (dry bones)
In darkness	40.03	13.30	22.13
In daylight + ultra-violet	38.88	13.49	22.06

In life there was no noticeable difference between the two groups. The growth curves of both males and females exceeded greatly the Donaldson



Rat G. 431. "Butter" Diet in Darkness. Age 84 days. Weight 160 grams. Rib.

- Co. Cortical bone, thick and well calcified.
- O. Physiological osteoid
- Sp. Primary spongiosa. Trabeculae numerous, thick, straight and well calcified.
- Z.P.C. Zone of provisional calcification well calcified.
- H.C. Hypertrophic cartilage cells.
- Z.C.P.C. Zone of proliferating cartilage cells.
- R.C. Resting cartilage.
- B.M. Bone marrow.

Diagnosis: Normal rib. Haem. and Eosin. Magn. $\times 40$.

Note: The ribs of all the rats were similar to the one illustrated above.

curve of normal growth for the same period of life. The rats living in darkness grew as quickly and reached as high an average maximum weight as those kept in daylight and exposed daily to the mercury vapour quartz lamp.

At autopsy there were no gross differences between the two groups. The eyes were normal and the teeth light yellow and transparent. All the rats were well nourished with an abundant deposit of fat in the natural regions. The musculature was well developed and of good colour. The chest was well developed, and there was no macroscopical abnormality in the ribs or long bones.

Histologically, the bones were normal in every respect, those of the two groups showing practically no differences (see Table I and Pl. IV).

The chemical analysis of the hind legs, carried out by Aron's method [1910], showed a very close agreement between the two groups. In no case was the variation in the percentage of calcium and of water in the bones of these groups greater than that found normally among rats from the same litter (see Table I).

The individual (Table I) and average (Table II) results are remarkably close for the rats kept in darkness and those that were kept in the light and in addition were irradiated daily with the mercury vapour quartz lamp.

SUMMARY.

Two litters of rats were placed on normal diets containing an optimal amount of calcium, phosphorus and the fat-soluble organic factor; in the diet of one litter this last was supplied by cod-liver oil, while for the other, two diets, one containing butter and the other cod-liver oil were used.

One-half of each litter was kept in the dark and the other in daylight with the addition of a daily irradiation for 20 minutes under a mercury vapour quartz lamp.

No appreciable difference was found between the growth of the two groups or histology and calcium content of the bones.

CONCLUSIONS.

Rats on a normal diet containing an optimal quantity of calcium, phosphorus, and fat-soluble organic factor, whether kept in darkness or in daylight supplemented by irradiation from a mercury vapour quartz lamp, grow equally well, and the development and calcification of their bones are normal.

We acknowledge our sincere thanks to Prof. C. J. Martin, for the privilege of carrying out the experiment at the Lister Institute.

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XXXV. A STUDY OF THE RELATION OF THE QUANTITY OF FAT-SOLUBLE ORGANIC FACTOR IN THE DIET TO THE DEGREE OF CALCIFICATION OF THE BONES AND THE DEVELOPMENT OF EXPERIMENTAL RICKETS IN RATS.

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(Received March 21st, 1923.)

INTRODUCTION.

As a result of the pioneer work of Mellanby [1918, 1919, 1920, 1921] on experimental rickets in the dog, it was thought at first that this disease might prove to be a deficiency one, caused, like scurvy and beri-beri, by the lack of a single organic factor from the diet, in this case the fat-soluble organic factor called vitamin A or fat-soluble A. Later the work of Korenchevsky on experimental rickets in the rat tended to confirm Mellanby's early findings and conclusions. In his first publication Korenchevsky states that the deficiency of vitamin A in the diet of rats "produces in some rats a picture resembling slight rickets, and typical rickets in some young rats whose parents had been fed on a diet deficient in vitamin A during conception, pregnancy and lactation." Deficiency of fat-soluble A, or of some other unidentified organic factor very closely associated with it, is now recognised as one of the important factors in the etiology of experimental rickets in dogs and rats. But, to date, most investigators of this subject who believe in the dietetic origin of the disease are of the opinion that the deficiency of this organic factor cannot be the sole cause of the disease. Some authors [Hess and Shipley and collaborators, McCollum and Simmonds, 1921] are very emphatic that a deficiency of fat-soluble A alone in a diet otherwise complete does not produce experimental rickets in rats but merely interferes with osteogenesis and causes the development only of osteoporosis. Similar conclusions were reached by Zilva, Golding, Drummond and Coward [1921], Tozer [1921] and Mackay [1921] as the result of studies on the effects of vitamin A deficiency alone in the diet of pigs, guinea-pigs and cats.

In his first communication on the subject Korenchevsky [1921] refers to

the fat-soluble organic factor as vitamin A, which is the term usually applied to the growth-promoting, anti-xerophthalmic, fat-soluble, organic factor. But in a monograph [1922] recently published he refers to it as the "anti-rachitic organic factor," without admitting or denying its identity with the growth-promoting factor. The work of McCollum, Simmonds, Becker and Shipley [1922] on the nature of the fat-soluble organic factor tends to show that the fat-soluble growth-promoting or anti-xerophthalmic factor and the anti-rachitic or calcium-deposition-promoting factor are either two distinct parts of the one substance or two separate substances very similar in their distribution and many of their properties. In the present communication, since a coincident deficiency of both factors was of necessity dealt with in the investigation, the inclusive term fat-soluble organic factor or simply organic factor will be used when referring to both, and the words growth-promoting or anti-rachitic will be prefixed to the term where specific reference is made to one or other of the factors.

At the time this investigation was started most of the results referred to above had not yet been published. The study was undertaken with a view to determine whether, as Mellanby found in the case of the dog, a deficiency of the organic factor alone in the diet can also produce rickets in rats, and if so, whether a relation exists between the quantity of this factor in the diet and the degree of calcification or severity of the pathological lesion of the bones.

EXPERIMENTAL.

PART I.

In this investigation the rats employed were bred in the Institute from animals fed on fresh cow's milk, white bread, oats and bran, green cabbage leaves (all *ad libitum*) and occasionally some red meat. The milk and cabbage leaf were the main source of the fat-soluble organic factor in this diet. The experiments were carried out during the latter part of autumn and during the winter of 1921, so that the milk was "winter milk," and probably not very rich in the organic factor. Judging from the rapidity with which the offspring from rats kept on the above diet showed the effects of deficiency of the fat-soluble organic factor in their diet, one must conclude that their ante-natal (and post-natal up to the time of weaning) supply of that factor was perhaps adequate but by no means abundant. On this account the same experiments were repeated with rats whose parents had received a larger supply of the organic factor before impregnation and during pregnancy and lactation. This was supplied to the parent animals in the form of cod-liver oil. The results of those experiments are being reported in Part III of this communication. All the rats were kept in a well-lighted and ventilated room, but away from the action of direct sunlight. The windows, which were kept closed, were of very thick plate glass.

Diets. The synthetic diet adopted for the experiments consisted of the following ingredients:

"Casein" (commercial)	20 g.	Marmite	5 g.
Starch (wheat)	50 g.	Orange juice	5 cc.
Fat	15 g.	Distilled water	50 cc.
Salt mixture ¹	5 g.		

This was kneaded into the form of a very thick paste, kept in tightly stoppered glass jars in a refrigerator, and was made fresh about twice a week.

It has been shown by Shipley, Park, McCollum and Simmonds [1921] that a diet which contains 3 % of butter, provided that diet is adequate in all other respects, especially the quality and quantity of its protein and the quantity and proportion of its inorganic elements, supplies an amount of the fat-soluble organic factor sufficient to permit of normal growth, reproduction and normal calcification of the bones of rats. For the normal control diet of these experiments the synthetic diet mentioned above in which the entire amount of fat consisted of fresh butter was adopted. This diet contained 15 g. of butter per 100 g. of dry constituents or 10 % of the wet paste as administered to the rats. This diet will be referred to as diet *A*.

In order to grade the amount of organic factor, yet retain exactly the same proportion of the various constituents, an ingredient had to be used which contained a moderate amount of this factor and which could be purified of it. In this connection advantage was taken of the work of Osborne and Mendel [1915] and of some unpublished work of Zilva (to whom thanks is due for the suggestion) who found that unpurified commercial "casein," when it forms 18 % of a diet otherwise complete, contains sufficient fat-soluble organic factor to permit of moderate growth at first and later slight gradual increase or maintenance of weight over a long period. But the weight curve is never normal and reproduction occurs but rarely. However, the development of pathological lesions in the bones of animals on this diet has not been reported. By using varying proportions of unpurified and inactivated² casein, the content of organic factor in the synthetic diet mentioned was graded without altering the quantity of the ingredient. Five diets differing only in their content of

¹ This is normal salt mixture No. 185 employed by McCollum [1917], the only difference being that the magnesium sulphate used was not anhydrous so that the amount of this salt was altered in order to keep the proportion of magnesium the same.

Salt mixture 185.

Sodium chloride	43.5
Magnesium sulphate	137.4
Sodium acid phosphate	87.2
Potassium phosphate (basic)	239.8
Calcium diacid phosphate	135.7
Calcium lactate	326.8
Ferric citrate	29.6
			<hr/> 1000.0

² Inactivated casein free from traces of the growth-promoting fat-soluble organic factor detectable by the biological method of Zilva and Miura [1921] was obtained by the heating and oxidation method [see Hopkins, 1920].

unpurified commercial casein, and therefore in their content of organic factor, were thus made. The fat used for these diets was hardened cotton seed oil free from organic factor.

Diet	% unpurified casein	% inactivated casein
<i>B</i>	20	0
<i>C</i>	15	5
<i>D</i>	10	10
<i>E</i>	5	15
<i>F</i>	0	20

In this way a rough gradation of the amount of fat-soluble organic factor was obtained. The difference between diets *A* and *B* in respect of their content of organic factor is obviously great, though how great, it is difficult to say. It was impossible to obtain sufficient animals from the same litter to place on diets with increasing amounts of organic factor so as to breach this gap between diets *A* and *B*.

In all six diets used in these experiments vitamin B (antineuritic) was supplied in the form of marmite and vitamin C (antiscorbutic) in orange juice¹.

The ash of these diets is alkaline. McCollum, Simmonds, Shipley and Park [1922] came to the conclusion that the reaction of the diet did not affect its rickets-producing qualities, but in a recent communication, Zucker, Barnett and Johnson [1923], have shown that for the diets they used, an alkaline ash made the diet more rickets-producing, and that by changing the reaction of a moderately rickets-producing diet to acid, they could convert it into a non-rickets-producing diet. What the effect of changing the reaction of the above diets would have been cannot be stated in this communication.

The amount of calcium in the above diets (*B*, *C*, *D*, *E* and *F*) was estimated by the McCrudden method and was found to be 0.258 g. per 100 g. of the wet paste (as administered to the animals). The normal diet (*A*) contained 0.285 g. per 100 g. of wet paste, slightly more than the others on account of the calcium contained in the butter included in that diet. The amount of phosphorus (estimated by Neumann's method) was 0.548 g. per 100 g. of the wet paste, and was practically the same for all the diets. Estimated for the diets without water the percentage of calcium was approximately 0.4 g. and phosphorus 0.8 g. per 100 g. of diet. When compared with the amount of calcium in the diets employed by McCollum and his collaborators, 0.64 g. per 100 g. of "dry" diet (as administered to the rats) and by Hess and Sherman and their respective associates, 0.48 g. to 0.55 g. per 100 g. of dry diet, the amount of this element used in the above diets is apparently low. Also, instead of the more usual calcium-phosphorus ratio of about two to one (the proportion by weight of these two elements in normal bone) the proportion of calcium to phosphorus in the above diets was approximately

¹ Since these experiments were begun it has been shown by Osborne and Mendel [1922] that orange juice contains also a certain amount of the growth-promoting fat-soluble organic factor. In a few tests by the biological method this was confirmed but the effect of the amounts contained in the diets was found to be very weak. However, in subsequent experiments (Parts II and III) decitrated lemon juice, apparently free from fat-soluble organic factor, was employed as the source of vitamin C.

one to two. Korenchevsky [1922], who employed the same salt mixture and in the same proportion as used in these experiments when studying the effect on rats of complete deficiency of "vitamin A" in the diet, came to the conclusion that the calcium content of the diet was probably low, but in the absence of definite knowledge of what is the optimal amount, and since this must vary with the amount of anti-rachitic factor in the diet, he decided that in "experiments designed to test the influence of varying amounts of anti-rachitic factor, the most striking results may be expected to arise when the content of calcium in the diet is not excessive." Nevertheless, as will be seen later on, although the amount of calcium in the diet was apparently low, yet in the presence of an adequate amount of the fat-soluble organic factor (diet *A*) normal calcification of the bones occurred and rickets did not develop.

In order, therefore, to ascertain that a double deficiency (calcium and organic factor) was not being dealt with, other experiments were performed using diets with increased amounts of calcium, and the results of these are reported in Part II of this communication. It may be stated in short that those experiments demonstrated that the amount of calcium used here was adequate, but by no means excessive and probably not optimal.

Twelve series of rats, every series consisting of six animals from the same litter were employed. Unfortunately, it was not possible to have all the animals of a series of the same sex. In all quantitative work of this kind, for proper comparison, it is best to have all the representatives of a series of the same sex, for it has been the experience of most investigators that males and females differ in their growth on normal diet and in their response to deficiencies in the diet. The twelve litters varied in age from 24 to 41 days at the time special feeding was begun. In the tables, the families are numbered I to XII. All the series were not killed at the same time because of uncertainty about the optimal period necessary for the manifestation of a pathological lesion. Six series were killed after seven weeks of special feeding, and the remainder at intervals up to fourteen weeks, only one series being allowed to remain on the special diets for so long a period (see litter XII in Table I). From every series a representative was placed on every one of the six diets *A* to *F*. Food was given *ad libitum*. The animals were fed daily and the amount consumed the previous day was recorded. The rats on normal diet "*A*" ate more than those on the various deficient diets, and their ration kept increasing as they grew older. But those on the deficient diets *B* to *F* ate larger portions during the first five weeks of the experimental period.

The average amount eaten during the entire experimental period by all the animals on the various diets was as follows:

Average amount consumed daily	
Diet	g.
<i>A</i>	14.3
<i>B</i>	11.2
<i>C</i>	9.7
<i>D</i>	9.9
<i>E</i>	9.3
<i>F</i>	9.4

The above figures do not apply to individual rats because in these experiments three animals on the same diet were kept in one cage. In the experiments reported in Parts II and III the exact amount eaten by every rat is known because they were kept in separate cages.

Growth on the different diets.

On normal diet *A* the rats grew well, though not quite as well as animals whose parents had received cod liver oil during pregnancy and lactation. Towards the latter part of the experimental period two of the animals (R. 347 and R. 352), females, did not gain the normal weekly amount. In those cases this was correlated by loss of appetite which developed for some unknown reason. The rats on diets *B* and *C* for the most part continued to gain steadily though slowly, but did not reach the normal figure. In a few cases, after from seven to eight weeks on these diets, the rats ceased to gain and only maintained their weight. On diets *D* and *E* the animals grew slowly and most of them reached their maximum weight in from five to seven weeks. A few continued to gain a little every week until they were killed. Those that did cease to gain were able to maintain approximately their maximum weight. The animals on diet *F* (completely deficient in fat-soluble organic factor¹) reached their maximum weight in from three-and-a-half to six weeks, depending upon the age and weight of the rat when it was placed on this diet. The older and heavier the rat when special feeding was begun, the more resistant to the deficient diet. All maintained approximately their maximum weight up to the time they were killed. Any indication of impending great loss of weight in one rat (loss of appetite or persistent slight decline of weight) was a signal to kill the whole series to which it belonged in order to avoid the complication of osteoporosis which starvation and loss of weight initiate in bones.

It can be seen in Chart 1 that the average maximum weight of the six series showed a gradation corresponding roughly with the gradation in the amount of organic factor in the diet. As mentioned before, the number of males and females in every group varied considerably so that the curve is not as striking as the indications are that it would have been, had all the animals been of the same sex. At *D* (Chart 1) this point is well illustrated. There is a rise in the curve although the content of the fat-soluble organic factor was less in this diet than in the one preceding it. But of the 12 animals on diet *D*, seven were male, while of the same number on diet *C*, only two were of that sex. Males generally reach a greater weight and gain more rapidly than females whether on normal or on deficient diets. This illustrates very well the fact that in all quantitative nutrition experiments, it is important for purposes of comparison to use animals of one sex in every series.

¹ Though diet *F* is referred to as being "completely" deficient in the organic factor, yet it is realized that undetectable traces were most likely present.

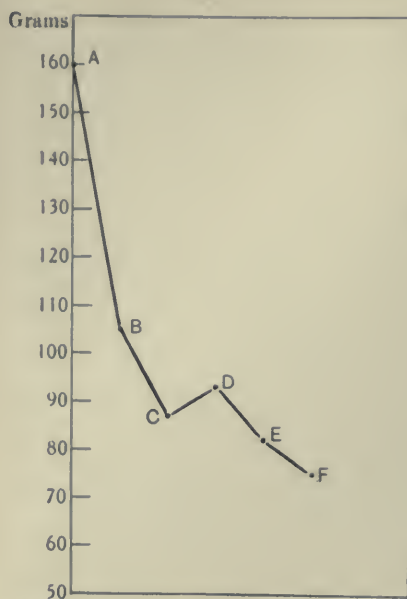


Chart 1. Average final weight of the rats on the different diets.

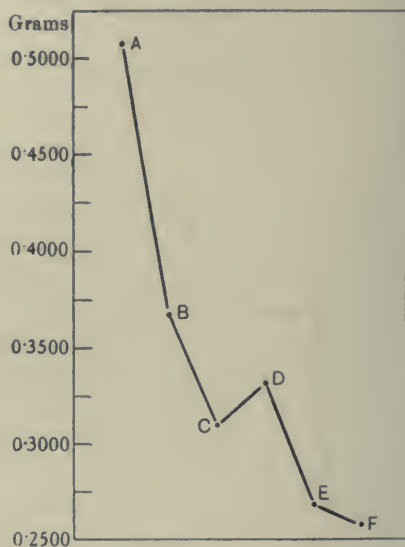


Chart 2. Average dry weight of bones (femur, tibia and fibula) of the rats on the different diets.

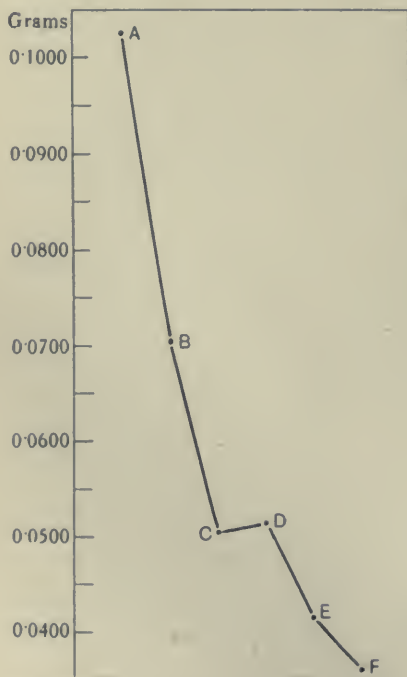


Chart 3. Average amount of calcium in the bones of the rats on the various diets.

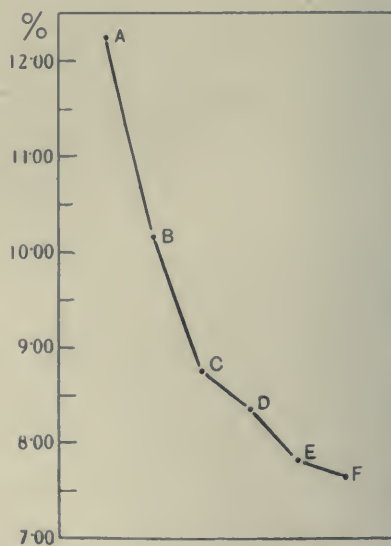


Chart 4. Average percentage of calcium per wet weight of bones.

Gross autopsy findings.

Although, as indicated in a previous part of this paper, the "store" of the fat-soluble organic factor in the rats used in these experiments was probably not very great at the time special feeding was begun, and although the calcium content of the experimental diets was probably not optimal, yet the control animals on diet *A* were, judging from gross appearances, quite normal. The fur was smooth and glossy, the eyes showed no abnormalities and the teeth in most cases presented the normal light yellow translucent appearance, though in a few rats there was a slight degree of opacity. The bodies seemed well proportioned, but no measurements were made. They were well nourished.

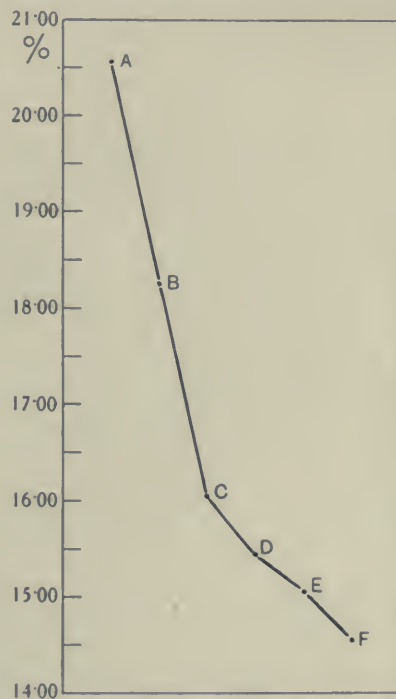


Chart 5. Average percentage of calcium per dry weight of bones.

There was an abundant deposition of fat in the normal regions, and the musculature was well developed and of good colour. The chest was full and rounded. The costal portion of the ribs was of the natural width and thickness for the size of the animals and, to the touch, appeared very well calcified. There were no deformities or fractures of any of the bones. The costochondral junction of the ribs was not enlarged, and the line of demarcation between cartilage and bone at the costochondral junctions was straight and very clearly defined. There was no epiphyseal enlargement of the long bones.

The animals on diet *B* differed grossly from the controls of the same litters on diet *A* in that they were smaller, lighter, and not as well nourished, though

better in all those respects than the rats on diets *C*, *D*, *E* and *F*, all poorer than *B* in their content of organic factor. The costal portion of the ribs was not as wide, as thick or as firm as that of the rats on diet *A*. In two rats (R. 367 and R. 369) several of the ribs showed a spontaneous fracture in the costal portion, but in no case was there any obvious deformity or enlargement at the costochondral junction of the ribs, or epiphyseal enlargement of the long bones. The shafts of the long bones were more readily broken than those of the normal rats.

The general appearance of the rats on diet *C* was similar to that of the animals on diet *B*, though they averaged a little smaller and lighter and were not quite so well nourished as the latter. The only striking difference was in the number of rats which had fractured ribs and the number of fractured ribs in those rats in which they occurred. The fractures occurred in four rats, affected from two to four ribs, and in one case there were several fractures in three ribs. In several rats there was slight enlargement of the costochondral junctions and the costochondral line was slightly curved and not quite sharply defined. The costal portion of the ribs and the shaft of the long bones were slightly more brittle than in those on diet *B*.

On diet *D* fractures were less frequent than in the rats on the foregoing diet. They occurred in two cases and were not multiple. There were no other striking differences between the animals on these two diets.

The rats on diet *E* presented a gross appearance similar in most respects to that of the rats on diet *F* to be described in detail below. They were slightly heavier, however, and a shade better nourished than those on diet *F*, but fractures occurred in the same number of cases (50 %), affected more ribs and were more often multiple.

The appearance of the rats on diet *F* was strikingly different from that of the animals on diet *A* from the same litters. They were very much smaller and lighter (about half or less) and were poorly nourished. The coat was rough and dull, and the front teeth in all cases were white and very opaque. This opacity of the teeth was always one of the first signs to develop as a result of deficient fat-soluble organic factor in the diet. All the animals on the above diets, with the exception of *A*, showed this to a more or less marked degree at the time they were killed. In no case did xerophthalmia develop. The chest in most of these animals was poorly developed, presenting the appearance of a tall cone with narrow base and, in many cases, sunken wall. Only in a few instances (rats R. 411, 413 and 415) were the costochondral junctions of the ribs greatly enlarged. In some there was no enlargement at all, while in the remainder this was only from slight to moderate. In 50 % of the animals on this diet fractures were present in many ribs, and in some the fractures were multiple. In both of these respects the condition was not quite as bad in the rats on diet *F* as in those on diet *E*. In the rats on both of these diets (*E* and *F*) the fracture was surrounded by a large, white, rather firm callus. The bony portion of the ribs varied in thickness and width

in the different animals, being very narrow and thin in some, and of moderate width and thickness in others, but in no case were they even approximately normal, with the exception of rat 418, which was placed on the deficient diet when aged 41 days and weighing 60 g. The costal portion of the ribs could be bent and broken with ease compared with the normal, and the same held true in most instances for the shaft of the long bones. In a few rats the lower epiphysis of the radius was obviously enlarged. This was also the case in some of the animals on diet *E*. In no case was there any evident deformity or fracture of the shaft of the long bones which could be recognised at autopsy. Histologically, however, one infraction of a radius from a rat on diet *E* was discovered.

The internal organs of the rats on the various diets showed no obvious gross pathology. In a few rats on diets *E* and *F* slight enlargement of the spleen was noted.

Histology.

Technique. From every animal, three or more ribs, one radius and one humerus were taken for histological examination. These were first fixed in formalin (10 % in normal saline) for 48 hours, and then subjected to slow decalcification in Mueller's fluid. In a few cases it was possible to cut the bones without previous decalcification. The latter, when sectioned, were treated with silver nitrate (the von Kossa method) and counterstained with alum, carmine and eosin. The sections of the bones which were partly decalcified in Mueller's fluid were stained with haematoxylin and eosin.

The microscopical appearance of the ribs and long bones of the animals on diet *A* was in most cases quite normal within the limits of natural variation. But in two rats (R. 347 and R. 352) a slight degree of osteoporosis complicated the picture. Both of these animals were gaining very little weight during the last two weeks of the experimental period. (For illustration of normal rib see Pl. V, figs. 1 and 2.)

In no rat on the various deficient diets (*B*, *C*, *D*, *E* and *F*) was the histology of the bones absolutely normal. But in a number of rats on the less deficient diets, especially diet *B*, the microscopical appearance of the bones was found to be nearly normal, and they are classified as such under the heading of diagnosis in Table I. These differed from the normal in that the cortex was thinner, the bone marrow cavity wider and the trabeculae of the primary spongiosa were fewer, thinner and less regular in their arrangement. But there were no cartilagenous changes and cortex, trabeculae and provisional zone of calcification were normally calcified. Thus a slight degree of osteoporosis complicated the otherwise normal picture.

Where a definite increase of cortical osteoid was present, without any abnormal changes in the cartilage, a diagnosis of osteomalacia was made, the degree varying with the amount of osteoid (see Plate V, fig. 4). It is realised that morphologically this cannot be distinguished from some cases of very late rickets, and that some authors now use as the only criterion for the

Table I.

No. of rat	Sex	No. of litter	Age (in days)		Days on diet		Weight (g.)		Gain in g.	In bones				Histological diagnosis
			Diet	Initial	Final	Initial	Final	Initial		% H ₂ O	% Ca	wet	% Ca dry	
R. 347	♀	I	A	32	81	49	38	110	72	43.66	11.60	20.33	20.33	Nearly normal (very slight osteoporosis)
R. 350	♂	II	A	34	83	49	47	177	130	40.95	12.52	21.20	21.20	Normal
R. 351	♀	III	A	30	79	49	41	177	136	40.53	12.00	20.18	20.18	"
R. 354	♀	IV	A	26	74	48	23	125	102	42.49	11.03	19.31	19.31	"
R. 356	♂	V	A	27	75	48	35	162	127	44.82	11.39	20.65	20.65	"
R. 357	♀	VI	A	24	72	48	21	137	116	41.83	12.03	20.69	20.69	"
R. 348	♀	VII	A	32	94	62	48	183	135	40.73	12.33	21.00	21.00	"
R. 352	♀	VIII	A	33	94	61	42	113	71	41.29	12.10	20.61	20.61	Nearly normal (very slight osteoporosis)
R. 353	♀	IX	A	25	86	61	22	165	143	41.96	11.83	20.39	20.39	Normal
R. 355	♂	X	A	26	88	61	25	126	101	40.40	11.86	19.91	19.91	"
R. 349	♀	XI	A	34	122	88	47	200	153	33.96	14.19	21.32	21.32	"
R. 358	♂	XII	A	41	137	96	61	247	186	33.94	13.95	21.56	21.56	"
Averages									40.54	12.23	20.59	20.59		
R. 359	♀	I	B	32	81	49	50	104	54	42.09	10.47	18.05	18.05	Nearly normal
R. 362	♀	II	B	34	83	49	42	93	51	43.96	10.91	19.47	19.47	"
R. 363	♀	III	B	30	79	49	33	103	70	45.92	10.34	19.17	19.17	"
R. 366	♀	IV	B	26	—	—	—	—	—	—	—	—	—	"
R. 368	♀	V	B	27	75	48	38	124	86	51.49	7.92	16.34	16.34	Died. Enteritis. Osteoporosis
R. 369	♀	VI	B	24	72	48	25	81	56	52.25	7.40	14.77	14.77	Moderate osteoporosis and very slight osteomalacia
R. 360	♂	VII	B	32	94	62	44	148	104	43.15	10.85	19.09	19.09	Moderate osteoporosis, slight osteomalacia
R. 364	♀	VIII	B	33	94	61	43	125	82	43.62	10.28	18.44	18.44	Nearly normal
R. 365	♀	IX	B	25	—	—	—	—	—	—	—	—	—	Very slight osteomalacia
R. 367	♂	X	B	26	88	62	27	91	64	45.93	9.49	17.56	17.56	Died. Enteritis. Severe osteoporosis
R. 361	♀	XI	B	34	122	88	42	96	54	41.13	11.15	18.95	18.95	Slight osteomalacia
R. 370	♀	XII	B	41	137	96	58	133	75	37.88	12.95	20.58	20.58	Nearly normal
Averages									44.74	10.17	18.24	18.24		
R. 371	♀	I	C	32	81	49	48	93	45	44.40	8.83	15.89	15.89	Slight osteomalacia
R. 374	♀	II	C	34	83	49	46	84	38	51.34	8.04	16.53	16.53	Moderate osteomalacia and slight osteoporosis
R. 375	♀	III	C	30	79	49	43	93	50	42.99	9.13	16.00	16.00	Very slight osteomalacia
R. 378	♀	IV	C	26	74	48	23	77	54	50.05	6.54	13.10	13.10	Moderate rickets
R. 380	♀	V	C	27	75	48	22	63	41	50.06	8.82	16.66	16.66	Very slight osteomalacia and osteoporosis
R. 381	♀	VI	C	24	72	48	22	70	48	53.25	6.58	14.09	14.09	Slight rickets and moderate osteoporosis
R. 372	♀	VII	C	32	94	62	43	108	65	36.50	10.62	17.76	17.76	osteomalacia
R. 376	♀	VIII	C	33	94	61	36	82	46	41.09	9.74	16.59	16.59	Moderate osteoporosis and slight osteomalacia
R. 377	♀	IX	C	25	86	61	25	65	40	48.33	6.28	12.16	12.16	"
R. 379	♀	X	C	26	88	62	26	82	56	46.18	8.98	16.74	16.74	Very slight osteomalacia
R. 373	♀	XI	C	34	122	88	48	108	60	38.04	11.05	17.84	17.84	Nearly normal
R. 382	♀	XII	C	41	137	96	54	127	73	40.74	11.25	19.00	19.00	"
Averages									45.26	8.78	16.03	16.03		

R. 383	♂	I	D	32	81	49	43	105	62	48.38	7.63	14.79	Moderate rickets and osteoporosis
R. 386	♂	II	D	34	83	49	50	110	60	44.09	9.55	16.88	Slight osteomalacia and osteoporosis
R. 387	♂	III	D	30	79	49	38	84	46	42.66	9.86	17.20	Moderate osteoporosis
R. 390	♂	IV	D	26	74	48	25	62	37	53.73	5.60	12.15	" rickets
R. 392	♂	V	D	27	75	48	33	80	47	50.75	8.21	16.68	Slight osteoporosis
R. 393	♂	VI	D	24	72	48	23	69	46	45.23	7.15	14.37	Moderate rickets and osteoporosis
R. 384	♂	VII	D	32	94	62	47	80	33	40.97	8.31	13.92	Very slight osteomalacia and osteoporosis
R. 388	♂	VIII	D	33	94	61	40	104	64	45.38	8.32	15.24	" rickets and moderate osteoporosis
R. 389	♂	IX	D	25	86	61	21	48	27	49.77	6.12	12.19	Moderate rickets and slight osteoporosis
R. 391	♂	X	D	26	88	62	30	96	66	48.46	7.64	14.83	" osteomalacia
R. 385	♂	XI	D	34	122	88	47	150	103	39.95	11.56	19.93	Nearly normal
R. 394	♂	XII	D	41	137	96	60	145	85	41.01	10.36	17.56	Slight osteoporosis
								Averages		45.86	8.36	15.47	
R. 395	♂	I	E	32	81	49	53	84	31	44.84	7.62	13.82	Moderate osteomalacia
R. 398	♂	II	E	34	83	49	40	74	34	48.95	9.01	17.58	Slight osteomalacia
R. 399	♂	III	E	30	79	49	49	111	62	48.59	8.25	16.08	Moderate osteomalacia and slight osteoporosis
R. 402	♂	IV	E	26	74	48	25	52	27	54.46	4.88	10.69	" rickets and osteoporosis
R. 404	♂	V	E	27	75	48	33	82	49	52.67	6.22	13.14	Slight osteomalacia
R. 405	♂	VI	E	24	72	48	20	49	29	55.70	4.98	11.58	Severe rickets
R. 396	♂	VII	E	32	94	62	50	101	51	44.40	9.10	16.51	Moderate osteomalacia
R. 400	♂	VIII	E	33	94	61	42	82	40	49.27	7.89	15.73	" rickets
R. 401	♂	IX	E	25	86	61	28	68	40	47.38	6.59	12.52	"
R. 403	♂	X	E	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 397	♂	XI	E	34	122	88	42	99	57	47.19	9.25	17.52	Slight osteoporosis
R. 406	♂	XII	E	41	137	96	57	120	63	41.69	12.20	20.92	Nearly normal
								Averages		48.65	7.81	15.09	
R. 407	♂	I	F	32	81	49	43	66	23	44.37	6.89	12.44	Very slight rickets and osteoporosis
R. 410	♂	II	F	34	83	49	38	75	37	45.63	9.50	17.37	Slight osteoporosis
R. 411	♂	III	F	30	79	49	44	109	65	48.85	7.52	14.69	Moderate rickets
R. 414	♂	IV	F	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 416	♂	V	F	27	75	48	33	63	30	48.80	6.33	12.37	Slight osteomalacia and moderate osteoporosis
R. 417	♂	VI	F	24	72	48	23	54	31	46.41	6.85	12.87	Very slight rickets and moderate osteoporosis
R. 408	♂	VII	F	32	62	30	—	—	—	—	—	—	Slight osteomalacia and moderate osteoporosis
R. 412	♂	VIII	F	33	94	61	49	62	13	44.25	7.46	13.11	Moderate osteoporosis
R. 413	♂	IX	F	25	86	61	27	58	31	53.27	5.80	12.44	Severe rickets
R. 415	♂	X	F	26	88	62	25	62	37	52.77	7.15	15.10	Moderate rickets
R. 409	♂	XI	F	34	122	88	43	77	34	49.05	8.19	16.00	Slight osteoporosis and very slight osteomalacia
R. 418	♂	XII	F	41	137	96	60	125	65	44.06	10.85	19.28	Slight osteoporosis
								Averages		47.75	7.65	14.56	

diagnosis of rickets, a definite increase in the amount of osteoid tissue regardless of the presence or absence of changes in the cartilage.

If, in addition to the increase of endosteal, periosteal and peritrabecular osteoid, pathological changes were also present in the epiphyseal cartilage or in that at the costochondral junctions, then a diagnosis of rickets was made, the degree depending "arbitrarily" upon the severity of the lesions. These lesions were the increased proliferation, disorganisation and imperfect calcification of the cartilage at the zone of provisional calcification, the presence and character of a rachitic metaphysis and the amount of osteoid. In some cases there were fractures, the callus of which consisted mainly of osteoid, poorly calcified or uncalcified cartilage and fibrous connective tissue (see Pl. VI, figs. 5-8 for varying degrees of rickets).

Both osteomalacia and rickets occurred in the rats on diets *C*, *D*, *E* and *F*. Both were in many cases complicated by some degree of osteoporosis and this is indicated under the heading of diagnosis in Table I.

The following table (Table II) shows a comparison of the number of normal, nearly normal, and pathological cases which occurred on the various diets. In this table they are classified according to the predominating lesion present, and in the case of osteomalacia and of rickets regard is taken of the severity of the condition.

Table II.

Diet	Total No. of Rats	Normal	Nearly normal	Osteo- porosis	Osteomalacia			Rickets			
					Very slight	Slight	Moderate	Very slight	Slight	Moderate	Severe
<i>A</i>	12	10	2	0	0	0	0	0	0	0	0
<i>B</i>	12	0	6	4	1	1	0	0	0	0	0
<i>C</i>	12	0	2	2	3	2	1	0	1	1	0
<i>D</i>	12	0	1	3	1	1	1	1	0	4	0
<i>E</i>	11	0	1	1	0	2	3	0	0	3	1
<i>F</i>	11	0	0	5	0	1	0	2	0	2	1

It is seen in the above table that rickets and osteomalacia were most frequent in the rats on diets *D*, *E* and *F* and that the two severe cases of rickets occurred in rats R. 405 and R. 413, on diets *E* and *F* respectively.

In the rats on diet *F*, pure osteoporosis occurred more frequently than in those on the other diets. Most investigators who have studied the pathology of the bones of rats on a diet completely deficient in fat-soluble organic factor have either killed the rats after they had been on the diet for a very long time, when, in many instances, they were losing weight very rapidly, or studied the bones of rats that had died after a long period on such a diet. It is probable that this fact accounts for the divergent results obtained and consequently for the difference of opinion held by investigators of the effect of deprivation of the fat-soluble organic factor alone from the diet of rats.

Analysis of bones for calcium content.

The calcium content of the bones was estimated by Aron's method [Aron and Sebaue, 1910]. Femur, tibia and fibula were chosen for analysis. These

were mechanically cleaned of all soft tissue with the exception of the ligaments round, and the cartilage and ligaments within, the knee-joint. The patella was removed. Each hind leg was analysed separately, but the percentage of calcium and of water corresponded so closely in the duplicate estimations that in Table I only the average of the two is given (see also Charts 2-5). The following table (Table III) gives the average figures for all the rats on the various diets.

Table III.

Diet	% Ca per wet weight of bones	% Ca per dry weight of bones	Deficiency of Ca as % of normal		% H ₂ O in bones	Excess of H ₂ O as % of normal
			Wet bones	Dry bones		
<i>A</i>	12.23	20.59	—	—	40.54	—
<i>B</i>	10.17	18.24	16.8	11.4	44.74	10.3
<i>C</i>	8.78	16.03	28.2	22.1	45.26	11.6
<i>D</i>	8.36	15.47	31.7	24.8	45.86	13.1
<i>E</i>	7.81	15.09	36.2	26.7	48.65	20.0
<i>F</i>	7.65	14.56	37.5	29.3	47.75	17.7

Table III shows a striking difference between the percentage of calcium in the bones of rats on diet *A* and that of the remaining five diets which differ from it only in the fact that their content of fat-soluble organic factor is less. In this table also a definite gradation of the average percentage of calcium in the bones is noted which corresponds with the gradation in the amount of fat-soluble organic factor in the diets (see Chart 5). The differences are not nearly as great between the remaining diets in respect either of calcium content of the bones or organic factor content of the diets. But there is as much or more difference between the calcium content of the bones of rats on diets *F* and *B* as between those on diets *B* and *A*. However, in the individual series (see Table I) this regular gradation is not always seen. The fact that the animals of a series were not always of the same sex probably interfered with the regularity of the gradation in calcium content, as it did in the case of the growth curves mentioned above.

When the amount of fat-soluble organic factor in the diet is adequate, the percentage of calcium deposited in the bones of rats of approximately the same age is remarkably uniform regardless of the weight of the animals, which may vary within fairly wide limits. At the age of about four months a rat on normal diet reaches its maximum degree of calcification.

On the other hand the rats on diets deficient in fat-soluble organic factor, especially those on diets *E* and *F*, showed very great variation in the calcium content of the bones, without any very definite relation to their final weight but with some relation to the age at which they were put on the deficient diets. The older and heavier the animal at the time it was put on the deficient diet the more likely it was to have a relatively high calcium content especially if not kept on the diet very long. (Compare rat R. 418 with R. 409 both on diet *F*, and R. 406 with R. 397 both on diet *E*.) The corresponding rats from

the same litters on the other diets more rich in organic factor showed less difference in their calcium content, despite the difference in the initial age, and, on the normal diet (*A*), rat R. 349 actually had a higher percentage of calcium in its bones than rat R. 358 despite the fact that the initial and final age of the former was less than that of the latter (see Table I).

The water content of the bones of rats on diet *A* was from 33 % to 42 % of the wet weight of the bones. In the animals on diet *F* the water content of the bones varied from 44 % to 53 %. The rats on diets *B*, *C*, *D* and *E* also showed a definite increase in the percentage of water. The water content of the bones increased as the amount of organic factor in the diet decreased (see Table II).

SUMMARY AND DISCUSSION.

A study has been made of the relation of the quantity of fat-soluble organic factor in a diet to the degree of calcium deposition in the bones and to the development of experimental rickets in rats kept away from direct sunlight.

By grading the amount of fat-soluble organic factor in the diet and leaving all other constituents constant, there resulted a corresponding gradation in the percentage of calcium deposited in the bones. It is, therefore, evident that the organic factor plays an important role in the mechanism for the deposition of calcium in bones, but how it acts is not elucidated by this investigation.

Korenchevsky found that with his "—A diet," which corresponds to diet *F* described above, he produced in some young rats a picture resembling rickets. This is confirmed by the results of the above investigation. What is more, it is shown that complete absence of the fat-soluble organic factor from the synthetic diet used is not necessary in order to initiate in the bones of some young rats changes which bear a definite resemblance to rickets or osteomalacia. In fact more cases of rickets and osteomalacia occurred in the rats on diets *D* and *E* than on diet *F*, which was the most deficient in the organic factor. This is perhaps best explained by the fact that on the latter diet complicating osteoporosis was more frequent and more severe, and this masked or prevented manifestation of the rachitic lesion.

Whenever the effect of the diminution or complete absence of only one of several variable factors is being investigated it is necessary that the others should be present in at least optimal quantity if not in excess. The three substances in the diet which, so far, have been considered to bear an important relation to the development of experimental rickets in rats are fat-soluble organic factor, calcium and phosphorus. Compared with the percentage used by other investigators the quantity of phosphorus in the above diets was certainly optimal. But the same cannot be said of calcium, for it was present in appreciably lower amounts than that recommended as the optimum by other investigators. McCollum and his collaborators, for the type

Table IV.

No. of rat	Sex	No. of litter	Age (in days)			Days on diet	Average amount of food eaten daily	Weight (g.)		Gain in g.	In bones			Histological diagnosis
			Diet	Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
G. 370	♂	XIII	F	27	83	56	10.4	42	94	52	46.95	7.83	14.80	Very slight rickets and slight osteoporosis
G. 371	♂	XIII	F	27	83	56	10.4	39	79	40	53.25	6.43	13.69	Moderate rickets
								Averages 50.10		Averages	50.10	7.13	14.24	
G. 372	♂	XIII	F 1	27	83	56	10.2	42	87	45	51.83	7.59	15.77	Very slight rickets and osteoporosis
G. 373	♀	XIII	F 1	27	83	56	10.2	38	90	52	51.64	6.92	14.32	Moderate rickets
								Averages 51.73		Averages	51.73	7.25	15.04	
G. 374	♂	XIII	F 2	27	83	56	11.6	42	95	53	48.10	9.81	18.90	Moderate osteoporosis
G. 375	♀	XIII	F 2	27	83	56	11.6	34	112	78	45.94	9.25	17.11	Slight osteoporosis
								Averages 47.02		Averages	47.02	9.53	18.00	
G. 376	♂	XIV	F	23	79	56	9.7	41	71	30	50.05	6.78	13.71	Slight rickets
G. 378	♀	XIV	F	23	79	56	9.7	43	76	33	48.80	6.77	13.23	Moderate rickets
								Averages 49.42		Averages	49.42	6.77	13.47	
G. 379	♀	XIV	F 1	23	79	56	9.4	41	80	39	44.14	8.12	14.60	Slight rickets
G. 380	♀	XIV	F 1	23	79	56	9.4	39	87	48	48.97	7.58	14.87	Moderate rickets
								Averages 46.69		Averages	46.69	7.85	14.73	
G. 381	♂	XIV	F 2	23	79	56	10.1	41	100	59	44.12	8.20	14.68	Slight rickets
G. 382	♀	XIV	F 2	23	79	56	10.1	36	82	46	45.95	8.30	15.36	Moderate osteomalacia
								Averages 45.03		Averages	45.03	8.25	15.02	
G. 339	♀	XV	F	27	78	51	9.4	31	56	25	54.30	6.53	14.28	Moderate osteomalacia
G. 340	♀	XV	F	27	68	41	9.4	33	54	21	48.20	5.72	11.50	Slight rickets and osteoporosis
G. 341	♂	XV	F	27	78	51	10.5	38	93	55	48.19	7.73	14.92	Slight osteoporosis
								Averages 50.23		Averages	50.23	6.66	13.56	
G. 342	♀	XV	F 1	27	78	51	11.7	34	87	53	43.83	8.90	15.84	Slight osteomalacia
G. 343	♀	XV	F 1	27	68	41	11.7	31	65	24	51.34	7.32	15.16	" rickets
G. 344	♂	XV	F 1	27	78	51	14.7	35	89	54	45.05	8.19	14.90	" osteomalacia
								Averages 46.74		Averages	46.74	8.13	15.30	

of diets used by them, recommend 0.64 g. per 100 g. of dry substance as the optimum for calcium, while in diets *A* to *F* used in this investigation the amount of calcium was 0.258 g. per 100 g. of wet paste or 0.42 g. per 100 g. of diet without water or orange juice. How close this quantity of calcium is to the optimal amount for the type of diet used cannot be stated, and without definite knowledge of the amount of this element absorbed from the diet by rats, cannot be correctly estimated. Therefore, an investigation was made of the effect of increasing the quantity of calcium in the diet most deficient in organic factor (diet *F*) used in the above experiments, and the result is reported in Part II of this communication.

PART II.

The effect of increasing the calcium content of diet F.

In order to ascertain whether, in the experiments described in Part I, one was dealing with a deficiency of calcium as well as of fat-soluble organic factor, the amount of calcium was increased in diet *F* (the diet most deficient in organic factor) and the effect on the bones of rats of the administration of this diet enriched in calcium content was investigated. To diet *F*, as used in the experiments of Part I, calcium carbonate was added so that two new diets were made, one with double and the other with four times the quantity of calcium contained in diet *F*, which will be referred to as diet *F* 1 and *F* 2 respectively.

Diet	Calcium per 100 g. of wet diet	Phosphorus per 100 g. of wet diet	Proportion of Ca to P
<i>F</i>	0.258	0.538	0.479 : 1
<i>F</i> 1	0.538	0.538	1 : 1
<i>F</i> 2	1.076	0.538	2 : 1

Experiments.

Series I. Of a litter of six rats similar in origin to those used in Part I, two were placed on diet *F* and the same number on each of the new diets, *F* 1 and *F* 2, containing increased amounts of calcium. They were kept on these diets for eight weeks. The results of chemical analysis and histological examination of the bones are given in Table IV.

Series II. Another litter of six rats was divided equally between the three diets. The results are summarised in Table IV.

Series III. Three rats of a litter of six were placed on diet *F* and three on *F* 1. The details of the experiment are given in Table IV.

The following is a comparison of the *average* percentage of water and of calcium in the bones of all the rats on the three diets.

Table V.

Diet	Average % Ca in wet bones	Average % Ca in dry bones	Average % H ₂ O in bones
<i>F</i>	6.95	13.85	50.39
<i>F</i> 1	7.54	14.89	49.21
<i>F</i> 2	8.89	16.51	46.02

An appreciable increase is noted in the average percentage of calcium per wet and dry weight of the bones, and a decrease in the average percentage of water in the bones of rats on diets *F* 1 and *F* 2 as compared with *F*. The highest average calcium content and the lowest water content was in the rats on diet *F* 2 which contained the greatest amount of calcium. But the differences between the three groups were not very great, and in all the rats the calcium content of the bones was very far below the normal, which, for rats of that age, should be about 13 % per wet weight and 21 % per dry weight. The water content of the bones should be about 38 % for normal rats of that age. Therefore, in all the rats of the three series the percentage of water in the bones is definitely increased.

The only gross differences between the rats on diets *F* 1 and *F* 2 and those on diet *F* were that the former averaged slightly larger and heavier (though their nourishment was no better) and fractures occurred less frequently. In the ribs of only one case on diet *F* 2 did fractures occur, in three on diet *F* 1 and in five on diet *F*.

Histologically, the differences between the bones of the three types of rats were not very great. In the main, the bones resembled those described for diet *F* in Part I of this communication. However, although the three types were distinctly abnormal, the bone lesions varying from pure osteoporosis to slight rickets (see Table IV) yet, in the main, the pathological condition was most severe in the rats on diet *F* and least in those on diet *F* 2. There was but little to choose between those on diets *F* and *F* 1 although the latter contained double the quantity of calcium, an amount practically equal to that considered optimal by McCollum and his collaborators.

SUMMARY OF PART II.

Although the increase of the amount of calcium in diet *F* so that it was optimal (diet *F* 1) and even excessive (diet *F* 2) resulted in a corresponding but not very great average increase of calcium deposit in the bones, yet it did not prevent the development of pathological lesions in the bones similar to those described for diet *F* in Part I of this investigation. From the above results one must conclude that the amount of calcium in diet *F* was adequate to protect against the development of pathological changes in the bones provided the other constituents of that diet were present in adequate amounts. Therefore, since the only known dietetic deficiency in diets *F*, *F* 1 and *F* 2 was the fat-soluble organic factor, the above results serve as further proof that a diet deficient in the fat-soluble organic factor alone and otherwise complete, when administered to rats without exposing them to direct sunlight or any artificial substitutes for it, can initiate in the bones of some young rats a pathological condition resembling rickets or osteomalacia.

PART III.

The effect of increasing the pre-experimental store of fat-soluble organic factor upon the degree of calcification and development of the bones of rats on diets similar to those used in Part I.

Coward, Lush and Palmer [1923], have shown that a rat stores an appreciable amount of the growth-promoting anti-xerophthalmic organic factor of its diet in at least one of its organs, the liver. The storage of this factor in the body of the rat has also been inferred from the indirect proof furnished by studies of the growth of rats from different litters on a diet completely deficient in the fat-soluble growth-promoting factor. The tremendous variation in the growth curves led to the conclusion that the store of this factor in the young depends upon the amount of it in the mother's milk and therefore, in the diet of the mother. It was suggested in Part I of this communication that the store of fat-soluble organic factor in the rats employed in those experiments was not very great at the time special feeding was begun, and that it was possibly a combined effect of slight deficiency of this factor during the pre-experimental period and the greater deficiency of it during the period of special feeding which was responsible for the poor calcification and the pathology of the bones.

Having determined (Part II) that the mere increase of the amount of calcium in the most deficient diet (*F*) employed in the experiments of Part I did not prevent the development of pathological changes in the bones, it was considered important to investigate the influence of the pre-natal and post-natal (up to the time the animals were put on the special diets) supply of the fat-soluble organic factor upon the effects produced by the administration of diets *B*, *C*, *D*, *E* and *F* described in Part I.

In order to do this, litters were bred from rats to whose general diet (similar to that of the parent rats described in Part I) was added a paste containing cod-liver oil as the extra source of the fat-soluble organic factor. This will be referred to as the "cod-liver oil breeding diet" and was constituted as follows:

Cod-liver oil breeding diet:

Commercial "casein"	20 g.	Tricalcium phosphate	4.8 g.
Starch	60 g.	Calcium lactate	0.3 g.
Cod-liver oil	5 g.	Potassium phosphate	0.6 g.
Yeast	6 g.	Water	67 cc.
Sodium chloride	0.3 g.		

Of this paste about 10 g. was given daily to every breeding rat prior to and during pregnancy, and the ration was increased up to about 25 g. during the period of lactation. As a result, these breeding rats, both male and female, were in excellent condition, reproduction occurred frequently, and the effect

on the young was very striking. At the age of from 18 to 21 days most of these young rats were as heavy as animals aged 25 to 28 days whose parents were on the same general diet but without the addition of cod-liver oil breeding paste. The young probably ate some of this special breeding paste for four or five days prior to the time they were put on experiment. With four litters of these specially bred rats, every litter consisting of seven females, the experiments described in Part I were repeated.

In order to give the extreme test to the prophylactic action of cod-liver oil in the parents' diet on the prevention of bone pathology in the young placed after weaning on the various diets deficient in organic factor (*B-F*), the rats were put on these special diets at the age of three weeks, much earlier than in the experiments of Part I. In the latter, the rats varied from 24 to 41 days, the average age at the time they were put on the special diets being 30 days.

The only changes were the substitution of decitrated lemon juice for the orange juice in all the diets, and the addition of a seventh diet, another normal control diet, containing 5 g. of cod-liver oil which replaced 5 g. of cotton-seed oil in diet *B*, but was otherwise similarly constituted. This diet will be referred to as diet "*AA*."

Diet "AA":

Starch (wheat)	50 g.	Salt mixture (No. 185)	5 g.
Unpurified "casein" (commercial)	20 g.	Lemon juice (decitrated)	5 cc.
Cod-liver oil	5 g.	Marmite	5 g.
Hardened cotton-seed oil	10 g.	Water (distilled)	50 cc.

From every litter a rat was placed on every one of the seven diets. The four litters were three weeks old at the time they were placed on the special diets, and they were all kept on them for nine weeks. This is a slightly longer period than was used in the experiments of Part I, but these rats felt the deficiency of the organic factor in the various diets so much less than those of the first investigation, that it was deemed advisable to allow them a longer period on the special diets in order to give the deficiency of the organic factor enough time to act.

The control rats on normal diets *AA* and *A* grew very well indeed, those on diet *A*, which contained butter, growing as well and looking as normal as those on diet *AA* which contained cod-liver oil.

The effect of the good reserve store of the fat-soluble organic factor was very noticeable, for all the rats on the various deficient diets, even those on the most deficient (diet *F*), gained weight more quickly, continued to gain for a longer period, reached a higher maximum weight and appeared more nearly normal than the rats on the corresponding diets in the experiments described in Part I. However, there was a definite difference between the growth of the rats on diets *AA* and *A* and that of the rats on the first deficient diet (*B*). But, unlike the results in the first investigation, very little difference

was noted in the growth and general condition of the rats on the five deficient diets. The gradation in the average maximum weight of the rats corresponding with the amount of organic factor in the diet which was noted in Part I did not manifest itself at the end of the experimental period. In fact, in a few instances the rats on diet *F* reached a maximum weight equal to that of some on diet *B*. The average final weight and average maximum gain differed but little in the rats on the five deficient diets but all were from 20 to 30 % less than the normal in those respects (see Table VI).

Macroscopical findings.

At autopsy, the rats on diets *AA* and *A* presented no gross abnormalities and were better nourished and developed than the rats on diet *A* in the first investigation. The butter used for diet *A* in the experiments of Part I was winter butter whereas summer butter was used in this investigation. The rats on the diet containing summer butter were similar in all respects to those receiving cod-liver oil in their food. Of course it should be noted that relatively much more butter than cod-liver oil was used, but the amount of cod-liver oil in diet *AA* was optimal.

The rats on the various deficient diets were not as large and not nearly as well nourished as the ones on the normal diets. Those on diets *B* and *C* from the same litters differed but little from one another, but on each of those diets the representatives from the four different litters did vary somewhat in their gross appearance. The teeth of some of these rats were slightly opaque, and the bony portions of the ribs and the shafts of the long bones were thinner and more brittle than normal. In one rat on diet *B* (G. 49) and one on diet *C* (G. 50) both from the same litter, there was very slight enlargement of the costochondral junctions. There were no fractures of any of the bones.

Two of the rats on diet *D* unfortunately died early in the experiment of acute enteritis. The remaining two (G. 31 and G. 97) were similar to those which showed the least abnormality on diets *B* and *C*.

Slight enlargement of the costochondral junction of the ribs was noticed in rat G. 32 on diet *E*, while the same enlargement, and fractures of several of the ribs were seen in rat G. 66. The bony portion of the ribs and the shaft of the long bones were quite brittle in these two cases. But the remaining two rats, with the exception of slight brittleness of the bones, showed no gross abnormality.

Two of the rats on diet *F* (G. 33 and G. 67) from the same litters as those on diet *E* which showed gross evidence of pathology of the bones, had moderate enlargement of the costochondral junctions and numerous fractures of the ribs, but rats G. 53 and G. 99 showed no gross evidence of disease of the bones except slightly increased fragility. In all the rats on diets *E* and *F* the teeth were moderately opaque, but the opacity was greatest in those rats which showed macroscopical lesions of the bones. It should be noted here that the

Table VI.

No. of rat	Sex	No. of litter	Diet	Age (in days)		Days on diet	Average amount of food eaten daily g.	Weight (g.)		Gain in g.	In bones			Histological diagnosis
				Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
G. 27	♀	XVI	A.A	21	88	67	16.0	20	155	135	39.39	13.80	22.86	Normal
G. 47	♀	XVII	A.A	21	84	63	15.5	24	168	144	35.99	14.18	22.16	"
G. 61	♀	XVIII	A.A	22	85	63	13.9	26	133	107	37.70	13.23	21.24	"
G. 93	♀	XIX	A.A	22	84	62	16.7	23	154	131	36.96	13.48	22.78	"
								Averages		Averages	37.51	13.67	22.24	
G. 28	♀	XVI	A	21	88	67	14.1	19	136	117	36.87	14.20	22.48	"
G. 48	♀	XVII	A	21	84	63	15.7	26	158	132	36.93	13.98	22.17	"
G. 62	♀	XVIII	A	22	85	63	15.5	27	171	144	39.02	13.73	22.52	"
G. 94	♀	XIX	A	22	84	62	15.9	22	134	112	39.59	13.82	22.87	"
								Averages		Averages	38.10	13.93	22.51	
G. 29	♀	XVI	B	21	88	67	14.6	20	128	108	40.33	12.37	20.73	Nearly normal
G. 49	♀	XVII	B	21	84	63	12.5	24	121	97	45.69	10.62	19.52	Very slight rickets (healing)
G. 63	♀	XVIII	B	22	85	63	11.0	31	101	70	40.95	10.43	17.67	Slight osteomalacia
G. 95	♀	XIX	B	22	84	62	15.5	22	123	101	38.70	12.18	19.87	Nearly normal
								Averages		Averages	41.41	11.40	19.44	
G. 30	♀	XVI	C	21	88	67	14.0	18	108	90	40.11	11.82	19.75	Nearly normal
G. 50	♀	XVII	C	21	84	63	13.1	23	127	104	43.60	10.70	19.13	Slight rickets
G. 64	♀	XVIII	C	22	85	63	13.1	29	112	83	41.90	10.17	17.49	Moderate osteomalacia
G. 96	♀	XIX	C	22	84	62	14.5	24	109	85	41.91	11.89	20.47	Nearly normal
								Averages		Averages	41.88	11.14	19.21	
G. 31	♀	XVI	D	21	88	67	10.4	20	77	57	44.01	9.16	16.36	Nearly normal
G. 51	♀	XVII	D	21	32	11	—	23	21	—	—	—	—	Died. Enteritis. Osteoporosis severe
G. 65	♀	XVIII	D	22	62	40	—	25	57	32	—	—	—	Died. Enteritis. Slight rickets, moderate osteoporosis
G. 97	♀	XIX	D	22	84	62	16.0	25	137	112	40.55	11.98	20.17	Nearly normal
								Averages		Averages	42.28	10.57	18.26	
G. 32	♀	XVI	E	21	88	67	16.3	20	110	90	40.82	12.0	20.28	Slight osteomalacia
G. 52	♀	XVII	E	21	84	63	12.9	24	97	73	41.82	10.95	18.82	Moderate osteoporosis
G. 66	♀	XVIII	E	22	85	63	9.9	23	78	55	47.16	8.52	16.12	Very slight rickets and osteoporosis
G. 98	♀	XIX	E	22	84	62	14.1	26	116	90	26.99	12.27	19.45	Nearly normal
								Averages		Averages	41.68	10.93	18.66	
G. 33	♀	XVI	F	21	88	67	14.7	19	96	77	42.95	9.82	17.21	Slight osteomalacia and osteoporosis
G. 53	♀	XVII	F	21	84	63	14.6	25	117	92	44.75	10.69	19.26	Slight osteoporosis.
G. 67	♀	XVIII	F	22	85	63	12.3	28	96	68	42.75	9.53	16.64	Slight rickets and osteoporosis
G. 99	♀	XIX	F	22	84	62	17.1	27	124	97	36.84	12.14	19.68	Nearly normal
								Averages		Averages	41.82	10.61	18.19	

gross lesions above described which manifested themselves in the rats on some of the deficient diets occurred mainly in those from the same two litters xvii and xviii.

Histology.

The bones of the control rats on diets *AA* and *A* were normal in every respect and no differences were noted between the bones of rats that received butter and those that received cod-liver oil in their food.

On diet *B*, the bones of rats G. 29 and G. 95 from litters xvi and xix respectively, were practically normal. The trabeculae of the primary spongiosa were not quite as large or as numerous and were not arranged as orderly as those of the perfectly normal bones, but the cartilage and cortex were normal. However, rat G. 49 from litter xvii had very slight rickets and rat G. 63 from litter xviii slight osteomalacia.

The rats which had diet *C* were similar to those on diet *B* from the corresponding litters.

Of the two rats on diet *D* which died early, one (G. 51) showed severe osteoporosis, and the other (G. 65) rickets and moderate osteoporosis. Rats G. 31 and G. 97 were practically normal, the histological picture being similar to that described for the bones of rats on diet *B* from the same litters.

In the bones of the rats on diets *E* and *F* osteoporosis was a more frequent complicating feature. All the animals on these two diets showed it in some degree (see Pl. V, fig. 3). One case of very slight rickets (G. 66) occurred among the rats on diet *E*, and one of slight rickets (G. 67) among those on diet *F*.

Chemical analysis.

As before, femur, tibia and fibula of both hind limbs were analysed for calcium content. Table VI shows that the bones of the rats on the control diets *AA* and *A* contained the normal amount of water and of calcium for rats of that age. No difference was found between the rats on the diet containing butter and those on the one containing cod-liver oil. In fact, on the average, the bones of rats on the butter diet calcified slightly better than those of rats on the cod-liver oil diet. The average calcium content of these control animals was slightly higher than that of the control rats on diet *A* in the experiments of Part I. Two facts probably explain this difference, (a) the greater store of the fat-soluble organic factor in the rats of the last experiments as compared with the first at the time they were placed on the special diets and (b) the fact that the butter used in the experiments of Part I was winter butter. Both undoubtedly played a part.

Contrary to what occurred in the experiments reported in Part I, no definite gradation was found in the percentage of calcium in the bones of rats on the various deficient diets. The bones of rats on diets *B* and *C* had practically the same percentage of calcium and of water, while those of the rats on diets *D*, *E* and *F* differed but little in their percentage of calcium and of

water. However, by dividing the seven diets into three groups, some indication of grading can be seen, the average percentage of water in the bones increasing, and that of calcium decreasing slightly as the content of the diet in fat-soluble organic factor diminished. As in the experiments of Part I, the greatest difference was between the normal and the first group of diets deficient in organic factor.

Table VII.

Diets	Average % Ca per wet weight of bones	Average % Ca per dry weight of bones	Deficiency of Ca as % of normal		Average % H ₂ O in bones	Excess of H ₂ O as % of normal
			wet bones	dry bones		
<i>AA</i> and <i>A</i>	13.79	22.35	—	—	37.80	—
<i>B</i> and <i>C</i>	11.27	19.32	18.28	13.56	41.64	10.15
<i>D, E</i> and <i>F</i>	10.70	18.37	22.41	17.81	41.93	10.92

SUMMARY OF PART III.

In order to determine whether a deficiency in the store of the fat-soluble organic factor in the body of the rats prior to the experimental period played a part in the results obtained in the experiments reported in Part I, the same experiments were repeated with rats whose parents had received cod-liver oil, before and during pregnancy, and the entire period of lactation.

The young grew better during the period of lactation, and later felt the effect of the deficient diets much less than the rats of Part I experiments, whose parents did not receive the special breeding paste containing cod-liver oil.

The average percentage of calcium in the bones of the rats on all the diets was higher and the percentage of water lower than on the corresponding diets in the experiments of Part I. There was very little difference between the percentages of calcium and of water in the bones of the rats on the various deficient diets, the gradation in those respects, so marked in Part I experiments, being very slight and apparent only when the diets were divided into three main groups *AA* and *A*; *B* and *C*; *D, E* and *F*. It would seem that the store of organic factor which these rats possessed at the time they were put on the special diets was able to make up in great part for its deficiency in those diets.

Despite the increased store of the fat-soluble organic factor which these rats presumably had at the time they were put on the special diets, one or two rats on all the deficient diets developed either slight osteomalacia or slight rickets. Those rats that developed a pathological condition of the bones were all from the same two litters *XVII* and *XVIII*.

PART IV.

Finally an investigation was made of the effect of increasing the calcium content of *F* diet, using rats bred from parents whose food contained an abundance of fat-soluble organic factor.

For this a litter of nine rats was employed bred from a mother who, in addition to the ordinary diet of fresh milk, white bread, oats, bran and green cabbage leaf, received daily for many months prior to and during the entire period of pregnancy and lactation, 10 g. of a paste containing fresh summer butter.

This will be referred to as "butter breeding paste" and was constituted as follows:

Butter breeding paste:

"Casein" (commercial)	20 g.	Calcium lactate	0.3 g.
Starch (wheat)	50 g.	Acid potassium phosphate	0.6 g.
Butter	15 g.	Yeast	6.0 g.
Sodium chloride	0.3 g.	Water	67 cc.
Tricalcium phosphate	0.8 g.		

The rats were only three weeks old, averaged 31 g. in weight, and were in excellent condition when they were put on the special diet.

By the addition of calcium in the form of calcium carbonate to diet *F*, diets *F* 1 and *F* 2 were made, the former containing double and the latter four times the quantity of calcium in the original diet *F*. These are identical with diets *F* 1 and *F* 2 used in Part II. Also, by removing some of the calcium lactate from salt mixture No. 185 and replacing this by powdered agar the calcium content of diet *F* was reduced by about one-fourth. This will be referred to as diet *F* 3. The purpose of the latter was to determine whether the calcium content of diet *F* is very near the lower limit.

Diet	Per 100 g. wet diet		Proportion of Ca : P	
	Amount of Ca	Amount of P	Ca	P
	g.	g.		
<i>F</i> 3	0.193	0.538	0.375	1.0
<i>F</i>	0.258	0.538	0.479	1.0
<i>F</i> 1	0.538	0.538	1.0	1.0
<i>F</i> 2	1.076	0.538	2.0	1.0

The rats were kept on these diets for eight weeks. All gained weight about equally, those on the diet containing the least amount of calcium (*F* 3) reaching a final weight as high as those of the corresponding sex on the remaining three diets. None reached the normal weight for rats of that age, but all grew better than those on diet *F* in Part I (whose parents were on the ordinary normal diet) (see Table I) and as well as those on diet *F* in Part III (whose parents were on the ordinary normal diet + cod-liver oil breeding paste (see Table VI)). At autopsy, no gross abnormalities of the bones were noted except slightly increased brittleness. In that respect the bones of rats on diet *F* 3 were slightly worse than the others, but no spontaneous fractures occurred in any of the rats. Histologically, no appreciable differences were found between the rats on diets *F*, *F* 1 and *F* 2, their bones being practically normal and showing only a very slight degree of osteoporosis. The bones of

Table VIII.

No. of rat	Sex	No. of litter	Age (in days)		Days on diet	Average amount of food eaten daily g.	Weight (g.)		Gain in g.	In bones			Histological diagnosis
			Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
G. 398	♂	XX	22	77	55	12.5	33	132	99	38.77	11.92	19.47	Slight osteoporosis
G. 399	♀	XX	22	77	55	12.5	31	107	76	39.55	12.15	20.12	Nearly normal
G. 400	♂	XX	22	77	55	13.1	31	97	66	40.05	10.23	17.09	Moderate osteoporosis
									Averages	39.45	11.43	18.89	
G. 401	♂	XX	22	77	55	14.5	33	116	83	39.88	11.85	19.72	Nearly normal
G. 402	♀	XX	22	77	55	14.5	30	117	87	38.75	13.29	21.21	" "
									Averages	39.31	12.57	20.46	
G. 403	♂	XX	22	77	55	13.2	31	139	108	40.65	12.09	20.80	Nearly normal
G. 404	♀	XX	22	77	55	13.2	30	92	62	38.50	12.12	19.71	" "
									Averages	39.57	12.10	20.25	
G. 405	♂	XX	22	77	55	11.4	31	108	77	39.80	12.32	20.49	Nearly normal
G. 406	♀	XX	22	77	55	11.4	28	83	55	36.87	12.40	19.65	Slight osteoporosis
									Averages	38.33	12.36	20.07	

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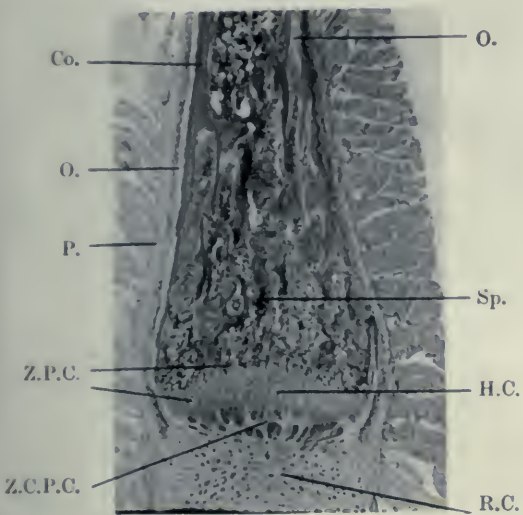


Fig. 5.

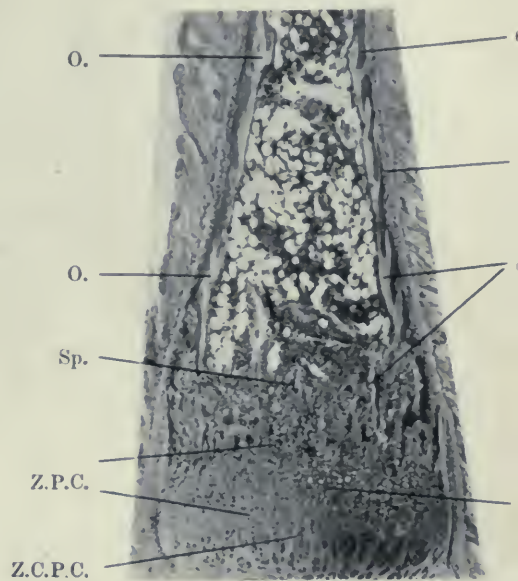


Fig. 6.

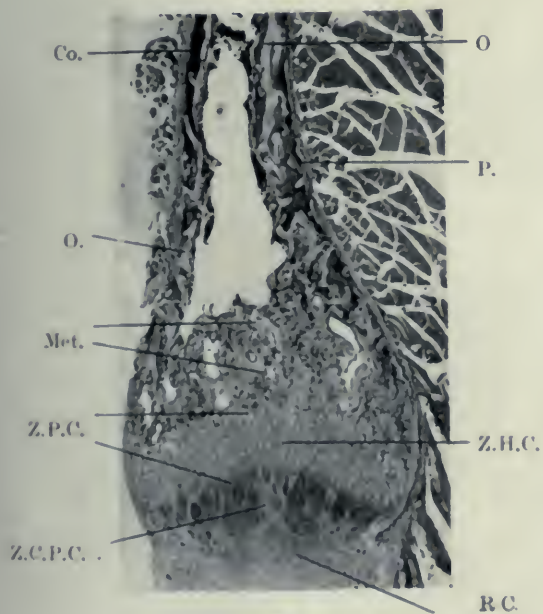


Fig. 7.

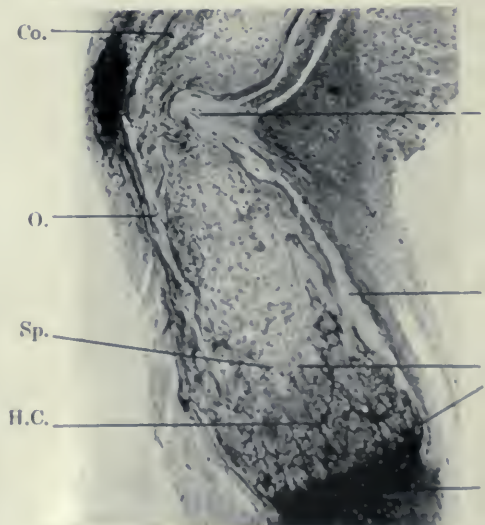


Fig. 8.

4. A normal diet containing 10 % (per wet weight) of fresh summer butter, proved as good as one containing an optimal quantity (3.6 %) of cod-liver oil, in causing normal growth and the normal development and calcification of the bones of rats.

5. The calcium content of the diets, although rather close to the lower limit, is adequate, but probably not optimal and certainly not excessive (Part II and Part IV).

Finally, it is a sincere pleasure to acknowledge my thanks to Prof. C. J. Martin and Prof. V. Korenchevsky for their kind interest and advice during the conduct of this investigation; to Miss K. M. Soames for very able technical assistance, and to Misses S. Rutherford, M. Pickersgill, and D. Russell for help in feeding and taking care of the animals at various times during the experiments.

EXPLANATION OF FIGURES.

The figures in Plates V and VI are from the rats of these experiments, and were chosen to illustrate the diagnoses given in the tables.

Abbreviations:

<i>Co.</i>	Calcified cortical bone.	<i>Z.P.C.</i>	Zone of provisional calcification.
<i>Sp.</i>	Spongiosa.	<i>H.C.</i>	Hypertrophic cartilage cells.
<i>O.</i>	Osteoid.	<i>B.M.</i>	Bone marrow.
<i>P.</i>	Periosteum.	<i>Met.</i>	Metaphysis.
<i>Z.C.P.C.</i>	Zone of proliferous cartilage.		

Fig. 1. Rat R. 350 (Part I). Costochondral junction of rib. Diet A. Normal Rat. Age 83 days. Weight 177 g. Normal calcification of cortical bone, trabeculae and zone of provisional calcification. Trabeculae of primary spongiosa numerous and regularly arranged. Very little osteoid. Normal rib. Haem.-eosin. Magn. $\times 32$.

Fig. 2. Normal rat. G. 27 (Part III). Rib. Description same as Fig. 1. Haem.-eosin. Magn. $\times 32$.

Fig. 3. Rat G. 52 (Part III, Table VI). Rib. Thin cortex. Very few trabeculae in primary spongiosa. Intense calcification of *Z.P.C.* Very little osteoid. Moderate osteoporosis. Haem.-eosin. Magn. $\times 32$.

Fig. 4. Rat G. 63 (Part III, Table VI). Rib. Note increase of osteoid. Zone of provisional calcification normally calcified. Slight osteomalacia. Haem. and eosin. Magn. $\times 32$.

Fig. 5. Rat G. 49 (Part III, Table VI). Note increase in size of zone of provisional calcification, the cells nearest the spongiosa showing fair calcification of their matrix. Increase of cortical osteoid and that surrounding the trabeculae of the spongiosa. Very slight (healing?) rickets. Haem. and eosin. Magn. $\times 32$.

Fig. 6. Rat G. 69 (Part I, Table I). Rib. Greater width, and imperfect calcification of zone of provisional calcification than in Fig. 5, rat G. 49. Also slightly more osteoid than in the previous figure. But cortex is relatively thinner and trabeculae fewer. Slight rickets and osteoporosis. Haem. and eosin. Magn. $\times 32$.

Fig. 7. Rat R. 389 (Part I, Table I). Rib. Note even greater width of zone of provisional calcification than in Fig. 6. Very imperfect calcification and some disorganisation of it by the ingrowth of blood vessels from the marrow. Relatively large amount of osteoid. Moderate rickets. Haem. and eosin. Magn. $\times 32$.

Fig. 8. Rat R. 413 (Part I, Table I). Rib. Note the very great width and imperfect calcification and disorganisation of the zone of provisional calcification. Relatively very large amount of cortical osteoid. Infraction of cortical bone, with deformity. Moderate rickets. Haem. and eosin. Magn. $\times 32$.

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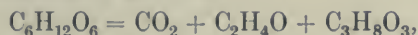
XXXVI. THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY DRIED YEAST IN THE SIMULTANEOUS PRESENCE OF PHOSPHATE AND SULPHITE.

By FUMIWO HEMMI.

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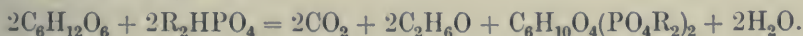
(Received March 23rd, 1923.)

NEUBERG has conclusively demonstrated in a long series of brilliant researches that when sugar is fermented by yeast, or by yeast preparations such as dried yeast or maceration extract, acetaldehyde is formed as an intermediate product and is then converted by reduction into alcohol. When the acetaldehyde is "fixed" by the addition of a sulphite, another intermediate product is reduced with the formation of glycerol,



and the fermentation is thus enabled to continue.

Now it has been shown by Harden and Young [1910] that in the normal fermentation of sugar by yeast preparations hexosediphosphate is formed according to the equation:



This is regarded by these authors as also representing the course of fermentation in the yeast cell, whereas Neuberger regards it as a pathological phenomenon which does not occur normally in the yeast cell, but only when the cell has been injured in some way, by drying, grinding, action of toluene, etc. Under these circumstances it was thought to be of interest to examine the effect of the presence of phosphate on the production of aldehyde by yeast preparations in the presence of sulphite, in order to ascertain whether either of these two changes in any way interfered with the progress of the other.

The results show that the addition of phosphate in no way interferes with the production of aldehyde. On the contrary the percentage of aldehyde, as calculated on the sugar actually fermented is usually slightly greater in the presence than in the absence of added phosphate. The fermentation of potassium hexosephosphate itself also yields aldehyde in presence of sulphite.

Further, the presence of sulphite does not prevent esterification of the phosphoric acid (see Exps. 5 and 6) so that the two processes in no way interfere. This is consistent with the view of Harden and Young that the phosphate is concerned in the early stages of the decomposition of the sugar molecule.

EXPERIMENTAL.

In order to obtain a series of comparable results dried yeast was employed in presence of toluene. Three different samples of dried yeast (Nos. 1, 2, 3) were used at different times. They were all prepared by pressing out fresh brewer's yeast at about $\frac{1}{2}$ ton per sq. inch and then drying at 37° for 48 hours.

The experiments were carried out by placing 5 g. of the dried yeast + 25 cc. H_2O + 0.2 cc. toluene + 1 (or sometimes 2) g. of the sugar in a flask and incubating at 30° in a thermostat, the CO_2 evolved being measured. After an interval, usually of 1 hour, additions were made in the form of 0.6M K_2HPO_4 , a solution of 20 g. $Na_2SO_3 \cdot 7H_2O$ per 100 cc. and if necessary water, all previously saturated at 30° with CO_2 . No estimations of p_H were made.

After the requisite period of incubation, during which any CO_2 evolved was measured, the contents of the flask were cooled in ice and made up to 100 cc. One portion of 50 cc. was filtered and 20 cc. taken for the estimation of inorganic phosphate by means of magnesium citrate solution and ammonia. The results are expressed as mgm. of K_2HPO_4 . In the remainder the acetaldehyde was estimated by the method described by Neuberg [1918]. Excess of barium chloride solution was added, the mixture allowed to stand in the cold, filtered and a portion of the filtrate distilled with $CaCO_3$ and the aldehyde estimated in the distillate.

Another portion (10 cc.) of the filtrate was taken for the estimation of reducing sugar by Bertrand's method, after precipitating the excess of barium by sodium sulphate.

The amount of sugar thus obtained does not however represent the total sugar which has escaped fermentation to CO_2 and either alcohol or aldehyde. In the first place any hexosediphosphate present at the close of fermentation would be almost completely precipitated by the barium chloride and in the second place any hexosemonophosphoric acid [Robison, 1922] would still be present, but would have a reducing power considerably less than that of free glucose or fructose. Further information can be obtained from a consideration of the amount of inorganic phosphate in the liquid, the difference between this and the total of the amounts added and originally present in the yeast representing phosphate combined either as hexosediphosphate or hexosemonophosphate.

In order to obtain an approximate idea of the amount of sugar fermented it has been assumed that the combined sugar is all present as hexosediphosphate.

In the first place it was found, in agreement with Neuberg's results [1919], that the addition of sulphite must be made after a vigorous sugar fermentation had commenced. When the sulphite was added along with the sugar at the commencement of the experiment, fermentation did not ensue, and even when the addition was made at the end of 30 minutes very little action occurred. When an hour was allowed to elapse before the addition was made,

satisfactory results were obtained. This is clearly shown in the following experiment.

Exp. 1. 5 g. of dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene, incubated at 30° .

To four series of flasks the following additions were made after intervals of 5, 30 and 60 minutes from the commencement of incubation and the mixtures were incubated for a further period of six hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2.5	2.5
2	0	2.5	7.5
3	5	5	0
4	0	5	5

Results.

No.	Time of addition mins.	Glucose mgm.		Free phosphate as K_2HPO_4 mgm.		Acetaldehyde mgm.
		Initial	Final	Initial	Final	
1	5	1000	1014	900	826	0
	30	916	923	880	791	5.1
	60	864	422	820	668	10.9
2	5	1000	1005	380	366	0
	30	916	970	360	383	4.5
	60	864	0	300	337	32
3	5	1000	1005	900	820	0
	30	916	920	880	781	8.3
	60	864	630	820	708	14.7
4	5	1000	1005	380	374	0
	30	916	978	360	381	7.7
	60	864	702	300	358	12.8

The initial figures are calculated from the amount of CO_2 evolved during the preliminary incubation.

In all subsequent experiments therefore the sulphite was added at least one hour after the fermentation had commenced.

It was also found that both phosphate and sulphite tended to stop the fermentation so that it was necessary to ascertain the best amounts to add. It was not found possible to use more than 5 cc. of 20 % sulphite (= 0.5 g. Na_2SO_3) + 10 cc. of 0.6M K_2HPO_4 (= 1.045 g. K_2HPO_4) with 5 g. dried yeast in a total volume of about 40 cc., and even this was excessive and resulted in a very small amount of fermentation. This only amounts to a concentration of sulphite of 1.25 g. Na_2SO_3 per 100 cc., whereas Neuberg (with living yeast) used 12.6 g. per 100 cc. This amount of sulphite is theoretically capable of combining with 174 milligrams of aldehyde corresponding with 0.73 g. of sugar fermented according to Neuberg's equation. In many experiments only 2.5 cc. of 20 % sulphite and 5 cc. of 0.6M K_2HPO_4 were used and in some only 1.25 cc. of sulphite.

The following is a typical experiment in which 5 cc. of sodium sulphite and 10 cc. of K_2HPO_4 were used.

Exp. 2. 5 g. dried yeast (No. 1) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene.

After 75 mins. incubation the following additions were made to two series of flasks.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	10	5	0
2	10	0	5
3	0	5	10
4	0	0	15

After the addition the flasks of Series 1 were incubated for three hours and those of Series 2 for six hours.

Results.

No.	Incubation period	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
			Total	Free	Combined	
1	3	476	1405	1105	300	10.1
	6	450	1405	1271	134	16.8
2	3	50	1405	1193	212	0
	6	0	1405	1304	101	0
3	3	512	360	310	50	9.1
	6	492	360	360	0	11.2
4	3	100	360	202	158	0
	6	0	360	228	132	0

The sugar present (free and combined) at the moment when the additions were made was 0.65 g. and the phosphate content of the dried yeast was 360 mgm. (K_2HPO_4).

In this case the amount of fermentation was greatly reduced by the addition of the sulphite, compare 1 and 2, 3 and 4. The amount of phosphate is also somewhat excessive.

Other experiments in which less sulphite was employed are the following:

Exp. 3. 5 g. dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene at 30°.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2.5	2.5
2	2.5	2.5	5
3	0	2.5	7.5
4	5	1.25	3.75
5	0	1.25	8.75
6	0	0	10

After one hour the total remaining glucose (free and combined) was 747 mgm. The analyses gave the following results:

No.	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	438	902	669	233	10.9
2	180	641	389	252	13.5
3	0	380	344	36	31.7
4	0	902	770	132	35.0
5	0	380	340	40	28.2
6	0	380	210	170	0

Here in presence of 5 cc. phosphate, 2.5 cc. of Na_2SO_3 solution has greatly diminished the fermentation, whereas in absence of the phosphate the sugar has been practically all fermented away as has also been the case in the presence of 1.25 cc. of Na_2SO_3 even in the presence of 5 cc. of phosphate.

On the assumption that the whole of the combined phosphorus is present as hexosediphosphate, the percentages work out as follows:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde	Acetaldehyde % of sugar fermented
1	309	121	188	10.9	5.8
2	567	130	437	13.5	3.1
3	747	19	728	31.7	4.3
4	747	68	679	35.0	5.2
5	747	21	726	28.2	3.9

It will be observed that both in presence of 2.5 and 1.25 cc. of sulphite the higher percentage is produced in presence of added phosphate. It must be remembered at the same time that the sulphite in 4 and 5 was only capable of combining with about one-third of the aldehyde theoretically obtainable from the sugar fermented.

Exp. 4. 5 g. dried yeast (No. 2) and 1.933 g. fructose (2 g. of 96.6 %) + 25 cc. H_2O + .2 cc. toluene.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0.6M K_2HPO_4 cc.	20 % $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ cc.	H_2O cc.
1	5	2.5	2.5
2	2.5	2.5	5
3	0	2.5	7.5
4	5	1.25	3.75
5	0	1.25	8.75

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	293	902	209	693	56.3
2	173	641	59	582	42.7
3	348	380	0	380	46.7
4	108	902	484	418	47.4
5	422	380	0	380	33.9

Treating these figures as those in *Exp. 3*, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1387	359	1028	56.3	5.5
2	1507	301	1206	42.7	3.5
3	1332	197	1135	46.7	4.1
4	1572	216	1356	47.4	3.5
5	1258	197	1061	33.9	3.2

Exp. 5. 5 g. dried yeast (No. 2) + 1.933 g. fructose (2 g. of 96.6 %) + 25 cc. H_2O + .2 cc. toluene at 30°.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2	3
2	5	2.5	2.5
3	0	2.5	7.5
4	5	3	2
5	0	3	7

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	72	902	675	227	62.7
2	87	902	458	444	74.2
3	231	380	153	227	66.0
4	216	902	311	591	65.3
5	159	380	15	365	51.1

Treating these figures as those in *Exp. 3*, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1608	117	1491	62.7	4.2
2	1593	230	1363	74.2	5.4
3	1449	117	1332	66.0	5.0
4	1464	306	1158	65.3	5.6
5	1521	189	1332	51.1	3.8

In No. 1 the amount of aldehyde is not far from the maximum capable of being combined with the sulphite present.

Experiments were also made to ascertain whether fructose, which reacts more readily than glucose with phosphate, would yield aldehyde more or less readily than glucose (*Exp. 6*) and whether hexosephosphate was also fermented with production of aldehyde (*Exp. 7*).

Exp. 6. 5 g. dried yeast (No. 3) + 25 cc. water + 0.2 cc. toluene and in (1) and (2) 2 g. glucose and in (3) and (4) 1.97 fructose.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2.5	0
2	0	2.5	5
3	5	2.5	0
4	0	2.5	5

Results.

No.	Residual sugar mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	0	902	773	129	75.8
2	0	380	363	17	50.9
3	0	902	794	108	74.9
4	0	380	371	9	53.8

Treating these figures as before:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde	
				mgm.	% of sugar fermented
1	1681	67	1614	75.8	4.7
2	1699	9	1690	50.9	3.0
3	1636	56	1580	74.9	4.7
4	1629	5	1624	53.8	3.3

Glucose and fructose appeared to act exactly alike. In both cases the percentage production in presence of added phosphate is appreciably greater than in its absence.

Exp. 7. In this experiment, in order to ascertain whether the decomposition product of hexosediphosphate had any special significance for the production of aldehyde, fructose and phosphate were added in such proportion that after the preliminary incubation the whole of the residual sugar should be present in the combined form.

The results obtained (summarised below) were of the same order as when free glucose or fructose was fermented.

No.	Time of incubation hours	Sulphite cc.	Sugar fermented calculated from hexosephosphate decomposed mgm.	Acetaldehyde	
				mgm.	% of sugar fermented
1 (a)	6	2	106	10.2	9.7
(b)	6	2.5	108	10.9	10.5
2 (a)	24	2	216	11.5	5.3
(b)	24	2.5	222	12.2	5.5

SUMMARY.

When the fixation method of Neuberg is applied to the fermentation by dried yeast of glucose or fructose, acetaldehyde is produced to about the same extent in the presence or absence of added phosphate.

I wish to thank Prof. A. Harden for advice and assistance during the prosecution of this investigation.

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XXXVII. THE RELATION BETWEEN URIC ACID AND ALLANTOIN EXCRETION IN HYBRIDS OF THE DALMATIAN HOUND.

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From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Committee.

(Received February 19th, 1923.)

THE research recorded in the present communication¹ was suggested by the remarkable fact, based on an observation made by Benedict [1916], that the Dalmatian breed of dog, *i.e.* the spotted coach dog, differs from other dogs in excreting a much higher percentage of uric acid. The late author thought, therefore, it would be interesting to ascertain how offspring from a cross between the Dalmatian and a normal dog would behave in this respect.

Uric acid and allantoin excretion in mammals.

In the group of mammals there are apparently three products of purine metabolism which may be excreted, *i.e.* uric acid, allantoin and purine bases. Though none of the three, so far as we know, is entirely absent from the urine, the proportion in which they are excreted varies considerably in different species of mammals. For the present purpose we are concerned only with the relative proportions of uric acid and allantoin. From the urine of man and the anthropoid ape, allantoin is almost, though not entirely, absent, whereas uric acid is present to a considerable extent. On the other hand, the urine of the dog contains very appreciable quantities of allantoin but small amounts only of uric acid. This variation in products is correlated with the existence, in certain tissues of the dog, of an enzyme which catalyses (and this may be demonstrated *in vitro*) the oxidation of uric acid to allantoin. This power of destroying uric acid is absent from the tissues of man and the higher apes. The capacity for destroying uric acid has been expressed by what is termed the "uricolytic index," that is the percentage ratio of allantoin nitrogen to the sum of allantoin nitrogen and uric acid nitrogen. The uricolytic index of the dog is about 98, that of man about 2. Of the majority of mammals investigated, the index is near 98, but in some it has been found to be as low

¹ The estimations included in this paper were carried out by Mr John Lowndes, assistant to the late Mr Onslow. Great credit is due to Mr Lowndes for his careful work and attention to the dogs, without which the research would not have been sufficiently complete for publication. (M. W. O.)

as 79 or 80 [Hunter and Givens, 1914; Hunter, Givens and Guion, 1914; Hunter and Ward, 1920]. As mentioned above, Benedict first discovered that the Dalmatian differs from the ordinary dog in regard to its excretion of uric acid, and this observation has been confirmed by Wells [1918]. Benedict observed that, on addition of acid to the urine of such a dog, a heavy precipitate of uric acid crystallised out. He found the high production of uric acid to be exhibited by four dogs of pure breed, but not by a fifth individual of doubtful breed. For the greater part of a year, a Dalmatian weighing about 10 kilos. was kept on a purine-free diet, and the uric acid determined almost daily. The following percentages and indices have been calculated from the data presented in Benedict's paper.

Table I.

Total nitrogen of urine in g.	Percentage of total nitrogen as		Uricolytic index	Remarks	
	Uric acid nitrogen	Allantoin nitrogen			
4.8	2.56	—	—	Diet contains 5.9 g. total nitrogen	
5.0	2.38	1.14	32		
4.9	2.57	1.31	34		
4.9	2.47	1.24	33		
5.1	2.35	1.00	30		
16.6	0.70	0.22	24	Diet contains 24.07 g. total nitrogen	
18.4	0.65	0.58	47		
19.8	0.65	0.52	44		
17.5	0.70	0.58	45		
15.2	0.76	0.55	42		
5.4	2.85	1.35	32	Diet contains 2.03 g. total nitrogen	
4.1	3.39	—	—		
3.4	4.09	2.00	33		
3.0	4.70	1.80	28		
2.8	4.96	2.11	30		
2.7	5.18	2.11	29	— —	
5.37	2.40	3.74	62		
5.65	2.23				
6.07	2.11				
5.52	2.26	3.56	62	Injection of 100 mgm. caffeine	
5.41	2.09				
5.23	2.22				
4.37	2.75	1.14	29	Normal diet	
4.54	2.75	1.10	29		
4.82	2.63	1.04	28		
6.48	2.73	3.24	54	Same diet + 100 g. thymus daily = 0.32 g. purine nitrogen	
6.86	2.87	3.06	52		
7.30	2.74	2.88	51		
5.75	2.24	1.01	31	Normal diet	
5.54	2.38	1.05	31		
5.27	2.60	1.10	30		
5.18	5.60	2.22	28	Same diet + 500 mgm. of uric acid subcutaneously	
5.13	5.83	2.77	32		
5.00	6.10	2.66	30		
5.02	2.47	1.31	35	— —	

From Table I we see that the uricolytic index of the dog used by Benedict varied from 24-62 according to the diet and other circumstances,

Object of research in the present paper.

As already stated, the object of the research recorded in the present communication was to ascertain how the lack of power of oxidation of uric acid was inherited in a hybrid animal produced by crossing the Dalmatian breed with a normal dog. For this purpose, a Dalmatian bitch (see Plate VII, fig. 1) of pure breed was obtained¹. She was subsequently mated with a large white terrier dog. Two hybrid offspring were born, a dog (see Plate VII, fig. 2) and a bitch (see Plate VII, fig. 3). Both had the build of a Dalmatian; the dog was entirely without spots, but the bitch was very slightly spotted with small black spots. A metabolism cage was used in the case of three of the dogs, but it was not found possible to put the Dalmatian in the cage.

Method of procedure and estimation.

The urine was collected in a vessel placed under the cage, and at the end of 24 hours the volume and specific gravity were taken. The floor of the cage was then carefully washed with distilled water, and the washings added to the urine. The mixture was acidified² with 5 cc. of 20 % sulphuric acid, and the whole made up to 500 cc. (250 cc. or less, in a few cases when the volume of urine was small). This dilution, together with the subsequent dilution when estimating the allantoin, ensures that the urea is not present to an extent greater than 1 %; otherwise it may be precipitated with the allantoin. This was verified by estimations of urea by the hypobromite and urease methods. It was also corroborated by calculating the percentage of urea likely to be present. This is estimated as 1.5 to 3 % as follows: In the dog, the total nitrogen, by volume, is 1 to 2 % of the urine³, and, of this, the urea nitrogen forms 75 % (i.e. 0.75 to 1.5 g. of urea nitrogen per cent., or 1.5 to 3 g. of urea in 100 cc. of the urine). The sulphuric acid was added, because allantoin is destroyed in alkaline solution, and the urine tends to become alkaline on standing [Givens, 1914]. It was not thought advisable to dilute the whole 24 hour specimen to the volume necessary for the allantoin estimation, as this would have caused the uric acid to be present in too great a dilution for accurate determination.

It was noticed, however, on acidifying some samples from the pure Dalmatian that a deposit of uric acid was formed, if allowed to stand, and might, in this way, be lost during the preliminary processes. Practically all these estimations, therefore, were carried out immediately, without acidification.

The uric acid was estimated by the method of Hopkins and Cole [Cole, 1920] and the allantoin by that of Wiechowski [1913]. All estimations were made in duplicate.

¹ From Mrs H. Wilson Bedwell, Heckfield, Hants.

² Except in the case of the Dalmatian as mentioned later.

³ In the case of the terrier recorded later, it was higher, due probably to the long period in the cage, i.e. 40 hours.

Estimation of total nitrogen. This was carried out according to Kjeldahl. As a rule, 5 cc. of urine, after the first dilution, were used.

Estimation of uric acid. Of the diluted urine, 300 cc. were taken (with one or two exceptions) and 60 cc. of 0.6 % colloidal ferric hydroxide were added which removes an unknown substance otherwise precipitated later by ammonium chloride. Duplicate 150 cc. portions of the filtrate were used. 2 g. of solid ammonium chloride were added for every 10 cc. of filtrate and stirred until dissolved, and then 3 cc. of strong ammonia. This was allowed to stand, after thoroughly stirring, for as long as 12 hours, when the uric acid was precipitated. The precipitate was filtered off, and washed with 10 % ammonium sulphate in 1 % ammonia solution to remove chloride. It was then washed into 100 cc. of hot water and 20 cc. of 45 % sulphuric acid were added. This was titrated with standard potassium permanganate solution, keeping the mixture at 65°, until the end point is noted by a pink flush which appears all through the liquid.

The amount of uric acid present can be calculated from the permanganate used. The results are expressed in terms of uric acid nitrogen, 1 cc. of $N/20$ permanganate solution being equivalent to 3.70 mgm. of uric acid.

Estimation of allantoin. To 100 cc. of the already diluted urine, 3 cc. of concentrated sulphuric acid and 7.5 cc. of glacial acetic acid were added, and the volume made up to 300 cc. Then excess of solid phosphotungstic acid was added (the necessary total amount having been previously calculated by using test portions of 2 cc. with 50 % phosphotungstic acid) and the whole stirred and allowed to stand about 12 hours. The precipitate was filtered off, and, to the filtrate, which was quite clear, lead oxide was added with constant stirring until the reaction was alkaline. The lead oxide, in the presence of acetic acid, forms both normal and basic lead acetates, and by these the phosphotungstic and sulphuric acids are removed and also certain constituents of the urine. The precipitate was filtered off, and the filtrate should give no further precipitate when tested with lead acetate solution. From 150 cc. of the filtrate the chlorides were then removed as follows. The liquid was made distinctly acid with acetic, and 1 % silver acetate solution was added until there was no further precipitate. The volume was made up to 200 cc. and allowed to stand some hours. It was then filtered, and sulphuretted hydrogen passed through the filtrate until the lead and mercury were completely precipitated. It was filtered again and the sulphuretted hydrogen removed by means of a current of air. Magnesium oxide was added until the filtrate was alkaline to litmus. It was finally filtered, and to 20 cc. of the filtrate the calculated amount of allantoin reagent (5 g. of mercuric acetate and 20 g. of sodium acetate made up to 100 cc.) was added. To determine the necessary amount of allantoin reagent, test portions were treated with the reagent until the latter gave no further precipitate. The filtrate from this, however, should give a flocculent precipitate with a very dilute solution of freshly-prepared allantoin. The precipitate of allantoin was

then filtered off and washed free from urea (the mercury compound of which is decomposed by water), until the washings gave no further precipitate with mercuric nitrate (test for urea). The nitrogen in the allantoin precipitate was estimated by Kjeldahl's method, using 10 % hyposulphite solution (2 cc. of 10 % of hyposulphite for every gram of mercury salt used). All estimations were made in duplicate.

Observations on the parents (Dalmatian and terrier).

It was not possible to put the Dalmatian into the metabolism cage, as she had not been trained and was, moreover, too large when the experiments commenced. Hence, samples of urine were collected at random. She was fed on a mixed and very liberal diet. When the experiments commenced on November 3rd, 1921, she was about 1 year and 8 months old. At the end of the experiments in 1922, her weight was 19 kilos (42 lbs.). The following is a record of the observations made for the Dalmatian and the terrier.

Table II.

Date of experiment	Volume of urine cc.	Sp. gr. of urine	In whole volume of urine			Uricolytic index	% of total nitrogen as	
			Total nitrogen g.	Uric acid nitrogen g.	Allantoin nitrogen g.		Uric acid nitrogen	Allantoin nitrogen
<i>Experiments with Dalmatian.</i>								
1921								
(1) Nov. 3	180	1.042	—	0.082	0.213	72	—	—
(2) Nov. 5	158	1.028	2.40	0.051	0.257	83	2.11	10.72
(3) Dec. 1 and 7	115	1.037	1.79	0.040	—	—	2.21	—
1922								
(4) Oct. 12	180	1.020	2.33	0.029	0.169	85	1.26	7.26
(5) Oct. 18	320	1.025	5.85	0.090	0.601	87	1.53	10.26
(6) Oct. 19	340	1.018	3.89	0.049	0.645	93	1.25	16.57
(7) Nov. 8	190	1.018	2.33	0.067	0.461	87	2.88	19.78
(8) Nov. 9	60	1.018	0.80	0.026	0.124	83	3.29	15.60
Average 84							2.08	13.36
<i>Experiment with Terrier.</i>								
1921								
(9) Nov.	110	1.040	3.67	0.010	0.703	99	0.26	19.97

Except in experiment 4, the urine was not acidified. In this experiment a certain amount of uric acid was precipitated on acidification, and this was filtered off, before adding colloidal ferric hydroxide, and estimated separately. There was no preliminary dilution of the urine in experiments 1, 2, 3 and 9. In experiments 4, 5 and 6, it was diluted to 500 cc.; in experiments 7 and 8 to 250 cc. and 150 cc. respectively.

From Table II it will be seen that, for the Dalmatian, the figures are in some respects very different from those calculated in the case of Benedict's dog. Though the results are vitiated in the present paper by the fact that 24 hour samples could not be obtained, it is obvious that a high percentage of uric acid excretion is possible, in fact crystals of uric acid were obtained on adding acid to the urine. The abundant diet is without doubt responsible for the high percentage of allantoin excretion. Umeda [1915] has shown that it may rise from 7 to 16 % on feeding with a diet rich in carbohydrate.

In the case of the Dalmatian, the greater the allantoin values, the higher will be the uricolytic index, whereas in the case of the normal dog, the uric acid value is so small that the allantoin may vary within wide limits without greatly affecting the value of the index. In any case it will be seen that the average figure for uric acid nitrogen expressed as a percentage of the total nitrogen is of the same order as in the case of Benedict's dog. It is much higher than what is found in normal dogs. The values should be compared with those obtained from the terrier with which the Dalmatian bitch was crossed and with those given by the hybrid offspring.

It was only possible to make one observation on the terrier. For this purpose he was kept in the cage for 40 hours. The results are apparently quite normal.

Observations on the offspring.

The two hybrid offspring were trained for the cage, and each experiment was of 24 hours' duration (with the exception of experiment 12 which only lasted 12 hours). They were fed upon the same diet as the Dalmatian. At the beginning of the experiments they were six months old. During the experiments the average weight was 13.6 kilos (30 lbs.) for the dog and 14.5 kilos (32 lbs.) for the bitch. The following is a record of the observations made on the two hybrid offspring.

Table III.

Date of experiment	Volume of urine cc.	Sp. gr. of urine	In whole volume of urine			Uricolytic index	% of total nitrogen as	
			Total nitrogen g.	Uric acid nitrogen g.	Allantoin nitrogen g.		Uric acid nitrogen	Allantoin nitrogen
<i>Experiments with Hybrid Dog.</i>								
1922								
(10) July 27-28	210	1.035	4.46	0.011	0.812	99	0.24	18.19
(11) Aug. 9-10	480	1.020	6.43	0.015	1.348	99	0.24	20.94
(12) Sept. 18	210	1.022	2.98	0.008	0.517	98	0.28	17.36
(13) Sept. 21-22	416	1.020	5.57	0.010	0.593	98	0.18	10.64
(14) Sept. 27-28	220	1.050	9.18	0.014	1.378	99	0.16	15.01
Average 99							0.22	16.43
<i>Experiments with Hybrid Bitch.</i>								
1922								
(15) July 31-Aug. 1	190	1.020	1.95	0.006	0.406	98	0.32	20.82
(16) Aug. 10-11	430	1.020	6.90	0.027	1.348	98	0.40	19.53
(17) Sept. 19-20	320	1.050	15.05	0.043	1.916	98	0.29	12.73
(18) Sept. 25-26	175	1.020	2.50	0.008	0.470	98	0.34	18.83
(19) Sept. 28-29	260	1.020	4.21	0.010	0.984	99	0.24	23.34
Average 98							0.32	19.05

The first dilution was in almost all cases to 500 cc. In experiment 12 it was to 250 cc. and in experiment 18 to 300 cc.

It was suggested by Mr S. W. Cole that the sulphuric acid used in acidification, might, as sulphate, interfere with complete precipitation of the ammonium urate. To test this, comparative estimations were made on the same sample, with and without acidification. The results were in complete accordance.

On the evidence of the above results, we may say that in the case of the hybrid Dalmatians the uric acid and allantoin excreted are in relatively the same proportions as in the normal dog. There is possibly a very slight excess of uric acid excreted by the slightly spotted hybrid bitch over the hybrid non-spotted dog. The greater power of destruction of uric acid is therefore a dominant character.

Sincere thanks are due to Professor Hopkins, F.R.S., for criticism and assistance with the writing of the paper, and to Mr F. W. Ward for advice on various points in connexion with the work. The photographs were kindly provided by Miss H. B. Killby.

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Fig. 1. Dalmatian bitch.



Fig. 2. Hybrid dog.



XXXVIII. THE HYDROGEN ION CONCENTRATION OF THE BLOOD IN CERTAIN PATHOLOGICAL CONDITIONS, AS DETERMINED BY THE HYDROGEN ELECTRODE AND THE INDIRECT METHODS OF BARCROFT AND HASSELBALCH.

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HASSELBALCH [1913] was the first to establish the conditions under which the hydrogen electrode can be used to measure accurately the reaction of the body fluids, and he constructed curves for the p_H of blood under varying CO_2 tensions.

More recently Peters [1914] has improved Hasselbalch's method by using completely reduced blood for the determinations, and Parsons [1917] has shown that by the C_H of blood is meant the C_H of the plasma it contains, i.e. the plasma obtained by centrifuging the blood without loss of CO_2 —which may be called the "true plasma" [Joffe and Poulton, 1920].

When once the hydrogen electrode apparatus has been set up the determinations do not occupy any great length of time; but as ordinarily arranged the apparatus itself tends to be somewhat complicated. A much simplified arrangement is, however, being designed by one of us (T. R. P.) especially for use with body fluids: it is soon to be placed on the market by the Cambridge and Paul Scientific Instrument Company. In this the manipulations are reduced to a minimum.

Barcroft's method [Barcroft and Peters, 1914] was introduced before the hydrogen electrode method had been finally perfected. It consists in determining at any required CO_2 pressure the percentage saturation with oxygen of the haemoglobin of the blood at a given oxygen pressure, and from this calculating the value of $\log K$ in Hill's equation. Barcroft and Peters [1914] showed for Barcroft's blood that $\log K$ was a linear function of p_H , and Hasselbalch [1916] found that in the case of six normal people the same relationship existed between the two values. Barcroft's method was used by Lewis, Ryffel, Wolf, Cotton and Barcroft [1913] in cases of cardio-renal disease, by Poulton and Ryffel [1913] in cases of uraemia, and by Poulton

¹ This work was carried out during the tenure of a Beit Memorial Research Fellowship.

[1915] in cases of diabetic coma. Hasselbalch [1916, 1917] has pointed out that even if the relationship between p_{H} and $\log K$ holds for normal people, it does not necessarily hold for pathological cases; but unfortunately he did not test the method against the hydrogen electrode, but by calculations from the concentrations of free and combined CO_2 in the blood. In this method of calculating the p_{H} of blood there is a slight systematic error as pointed out by Parsons [1917].

1. METHODS.

Collection of blood, etc. The blood was obtained by venous puncture from patients in Guy's Hospital at about 11 a.m. It was immediately defibrinated, packed in ice inside a vacuum vessel, and sent by passenger train to Cambridge where it was received by T. R. P. at about 2 p.m. The determinations were carried out at once except in cases specially mentioned. The following investigations were made: (1) the p_{H} of this fully reduced blood at 37° at three or four CO_2 pressures by the method described by Parsons [1917]; (2) the CO_2 content of the same specimen of blood by means of Barcroft's differential apparatus; (3) the $\log K$ at the same temperature at varying CO_2 pressures, also with Barcroft's differential apparatus.

The alveolar CO_2 was obtained, by E. P. P. at the time that the blood was taken, by a modification of Hasselbalch and Lindhard's method which is described by Campbell, Hunt and Poulton [1923]. In this series of cases the patient, wearing Bohr's mask and valves, was told to expire deeply towards the end of his normal expiration. Inspiratory samples were not usually taken. In some of the earlier cases the ordinary Haldane-Priestley method was used.

In a few cases E. P. P. determined the CO_2 in the fully or partly oxygenated blood with Van Slyke's apparatus, the CO_2 pressure being measured as described in Joffe and Poulton's paper [1920]. A few determinations of $\log K$ were also made by him, according to Barcroft's original method [1914].

2. THE RELATION BETWEEN $\log K$ AND p_{H} IN PATHOLOGICAL BLOODS.

This relationship has been carefully tested with the normal blood of Barcroft [1914] and with Parsons' blood, with the addition of lactic acid [Donegan and Parsons, 1919] and by Barcroft and co-workers [1922]. In Fig. 1 the two lines indicate this relationship for the two bloods. The lines are parallel to one another; but they are obviously quite different. Thus at a p_{H} of 7.4 which is a normal p_{H} for reduced arterial blood, the $\log K$ for Barcroft's blood is 4.575, for Parsons' lactic acid blood it is 4.295. The few determinations carried out on Parsons' normal blood show that the $\log K$ - p_{H} relation is the same as for the lactic acid blood. The difference between these two lines is far outside the experimental error; it is to be partly accounted for by the fact that the p_{H} values obtained by Peters for Barcroft's blood are appreciably more acid than those obtained by Parsons working with his own blood. But Peters was more concerned with the differences in p_{H} produced

in blood under various physiological conditions than with the absolute value of the p_{H} at any time, and does not claim to have established this absolute value more than approximately in any given case. The average difference between Peters' result at any given pressure of CO_2 and that obtained by Parsons at the same carbon dioxide tension is about 0.1 in the value of the p_{H} , which is approximately half the difference between the p_{H} values corresponding to a given value of $\log K$ on the Peters-Barcroft curve and that found by Donegan and Parsons [1919]. It is to be noted, however, that Hasselbalch [1916] obtains values for normal blood which coincide with the original Peters-Barcroft curve, but he used p_{H} values calculated from the carbon dioxide content of the whole blood.

The $\log K$ - p_{H} relationship was tested on 16 cases (see Table I) and the results may be compared with Parsons' lactic acid blood curve in which the $\log K$ at $p_{\text{H}} = 7.4$ is $\bar{4}.295$ and with the P.-B. curve in which the $\log K$ at $p_{\text{H}} = 7.4$ is $\bar{4}.575$.

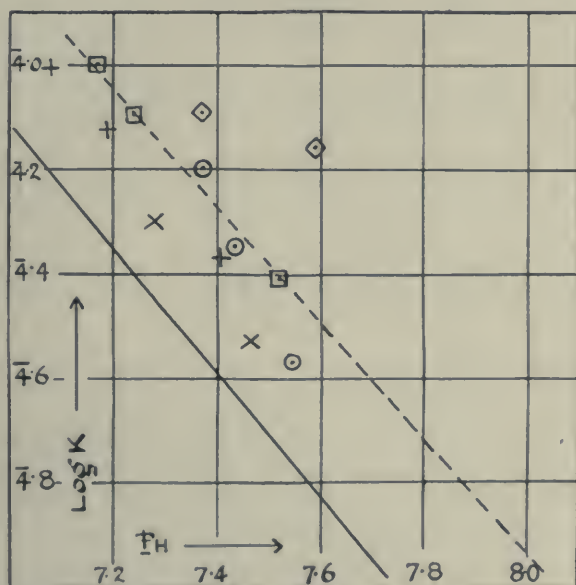


Fig. 1. $\log K$ - p_{H} curves for typical cases:

- | | | | |
|------|--|---|-------------------------|
| + | Case 4 (azotaemic nephritis). | ◇ | Case 11 (cardio-renal). |
| □ | „ 6 (polycythaemia). | ○ | „ 12 (mitral stenosis). |
| x | „ 10 (diabetes). | — | P.B. line. |
| ---- | Log K - p_{H} curve obtained with Parsons' blood to which 0.04 % of lactic acid had been added. | | |

Some typical examples of the relationship are shown in Fig. 1. In cases 4, 6 and 10 the lines joining the points are parallel with the curve for Parsons' blood and the P.-B. line, while in cases 11 and 12 there is no parallelism. Summarising our results, out of 21 examinations on 14 cases, there are six in which there is no parallelism, whereas in 18 the parallelism is fairly close.

There was no parallelism in a case of tabes (15) where the blood had been kept overnight, but had not apparently developed much acid; in a cardio-renal case (11); in tubal nephritis (8) on one occasion, but not on another; in mitral stenosis (12); in diabetic ketosis (9) on two occasions out of three, on one of which the blood had been kept, and on these two occasions the lines were not parallel to each other. It may be said that there is usually parallelism, but we cannot find any reasons for the exceptions.

In Table I (last column) we have defined the relationship by the value of $\log K$ at p_H 7.4. Analysing these 23 results (two of which are rather doubtful, as only single $\log K$ determinations were made) we find that they group themselves roughly about the curve for Parsons' blood. Thus eight results are greater than $\bar{4}.34$ and eight are less than $\bar{4}.28$, while seven are intermediate and the mean of these seven is $\bar{4}.30$, which is practically the same as for Parsons' blood ($\bar{4}.295$).

In cardio-renal disease and mitral disease with heart failure the $\log K$ at p_H 7.4 tends to be low, the mean value for cases 2, 16, 11, 12, 14 and 7 being $\bar{4}.23$. This may have some bearing on Lewis, Ryffel, Wolf, Cotton and Barcroft's [1913] determinations of $\log K$ in cases of breathlessness. These authors found that the $\log K$ was low and considered that this was due to the presence of fixed acid in the blood in abnormal amounts, which produced an acidaemia. In Campbell, Hunt and Poulton's [1923] paper it has been pointed out that the fixed CO_2 of the blood is hardly diminished at all in these cases. Our present results suggest that there is a distinct tendency in cardiac cases for the $\log K$ to be low when compared with the p_H . This might possibly explain Lewis and Barcroft's findings; for, out of 13 determinations on the special cases showing meionexy in their paper there are only five in which the p_H at 40 mm. would be more acid than 7.40, supposing that $\log K$ was $\bar{4}.10$ at p_H 7.40, as in our case of myocardial degeneration (case 11). In making this estimate we have calculated $\log K$ at 40 mm. CO_2 assuming that alteration of $\log K$ with CO_2 pressure was the same as in Parsons' lactic blood.

In three cases of diabetes with much ketosis $\log K$ at p_H 7.4 tended to be greater than in Parsons' blood, sometimes reaching the P.-B. curve, as follows: case 9 (lipaemia) $\bar{4}.32$ on the first occasion; $\bar{4}.85$, i.e. beyond the P.-B. curve on the second occasion when the blood was kept overnight; $\bar{4}.27$ on the third occasion; case 10, $\bar{4}.45$; case 13, $\bar{4}.64$. In order to draw conclusions as to the p_H of blood from determinations of K in six cases of diabetes verging on coma or actually comatose, Poulton [1915] used the P.-B. curve for his calculations of p_H from $\log K$. Our present results indicate that this possibly gave correct results at any rate in some of the cases.

In five cases we have determined the $\log K$ - p_H relation on more than one occasion. In cases 4 and 6 (azotaemic nephritis, and erythraemia) there was no alteration before and after treatment in the oxygen chamber; but in case 4 $\log K$ at p_H 7.4 changed from $\bar{4}.44$ to $\bar{4}.35$ in three months, while the fixed acid in the blood increased considerably. On the other hand in case 5 (normal)

the fixed acid apparently increased during oxygen treatment but there was no alteration in $\log K$. There was some slight alteration in the slope of the $\log K$ - p_H curve and of the $\log K$ value at p_H 7.4 in case 8 (tubal nephritis, lipaemia) after six weeks. Case 9 (diabetes with a marked ketosis) showed great changes in the relation within the space of a month.

It is of course unsafe to generalise on a few cases; but our results would appear to suggest that in diabetes with severe ketosis $\log K$ tends to be rather high for a given value of p_H . On the other hand in cardiac disease with circulatory failure, including valvular disease and myocardial degeneration, $\log K$ tends to be rather low.

3. THE COMPARISON OF THE p_H OF BLOOD CALCULATED FROM THE CO_2 OF THE BLOOD WITH THE p_H DETERMINED BY THE HYDROGEN ELECTRODE.

It has been pointed out by Parsons [1917] that the p_H of blood means the p_H of the "true plasma." Consequently the most direct way of comparing Hasselbalch's CO_2 method with the hydrogen electrode would be to determine at a given CO_2 pressure the CO_2 content of the true plasma and from this to calculate the p_H by means of the formula [see Joffe and Poulton, 1920], and also to measure the p_H of the same specimen directly by the hydrogen electrode. In the case of reduced blood it is unnecessary to use the "true plasma" for the electrode measurement, as the same results are obtained with whole blood.

We have made determinations of this kind in two cases of myocardial degeneration (Figs. 2 and 3). In case 16 the p_H (observed) of the oxygenated plasma was less than that of the reduced blood as would be expected from Parsons' work [1917]. The calculated values from the CO_2 of the plasma agree fairly well with these results, as shown in Fig. 2. [For some unexplained reason the values calculated from the CO_2 of the blood are much too low in this case.] In case 17 (Fig. 3) we have five calculations from the CO_2 of the plasma, and two calculations from the CO_2 of the blood, but, unfortunately, only two values for the observed p_H . The correspondence between them is fair. Further observations of a similar nature would be desirable before it would be possible to say that the truth of this proposition had been conclusively demonstrated.

In case 13 (diabetes with ketosis) we have determinations of p_H both for the reduced blood and oxygenated true plasma. Parsons found that the average difference between the p_H of his oxygenated and reduced blood was 0.038. The differences in the case of the patient's blood were 0.03 and 0.035, agreeing fairly well with Parsons' figures. This difference of course depends primarily on the fact that oxyhaemoglobin is a stronger acid than reduced haemoglobin; and it is satisfactory to be able to conclude that in a pathological state such as diabetes the same theory also holds good.

In all our calculations we have used the Hasselbalch formula [1916] with a constant value of 6.096 for p_{K_1} , according to Warburg's recommendation

[1922, p. 207]. In calculating the free CO_2 we have used Bohr's solubility coefficient for CO_2 in serum, viz. 0.541 [Hasselbalch, 1916].

The true significance of the CO_2 content of true plasma had not been realised during the earlier part of the work, and although we made many determinations of the CO_2 content of whole blood the CO_2 of the "true plasma" was not determined. However, Campbell, Hunt and Poulton [1923] have

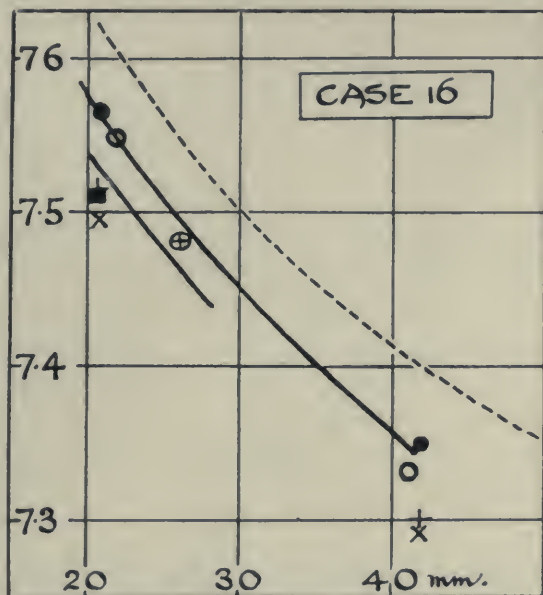


Fig. 2. The dotted line represents the normal relation between p_{H} and CO_2 tension in the reduced blood of T. R. P.

The points indicate values of this relationship in the blood of case 16, obtained as follows:

- By direct electrometric measurements on the completely reduced blood (T. R. P.).
- × By calculation from the CO_2 content of the completely reduced blood (T. R. P.) using the blood-true plasma CO_2 curve.
- + By calculation from the CO_2 content of the completely reduced blood, using Warburg's method.
- By calculation from the CO_2 content of the true plasma of the partly oxygenated blood (E. P. P.).
- By electrometric measurement on the true plasma of the fully oxygenated blood (T. R. P.).
- ⊕ By calculation from the CO_2 content of the true plasma of the fully oxygenated blood (E. P. P.).

determined the CO_2 both in whole blood and in the corresponding true plasma in quite a number of pathological conditions. In Fig. 1 of their paper, where these two values are plotted at right angles to one another, the results fall on a straight line, provided the haemoglobin content of the blood is about normal. Using their diagram we have calculated the CO_2 of the true plasma corresponding to our determinations in whole blood, and these plasma results have then been used for calculating the p_{H} by Hasselbalch's formula. This has been carried out for all our determinations of CO_2 in blood. It has been thought advisable to make one small correction. Campbell, Hunt and Poulton's curve

applies only to oxygenated blood, while we are dealing here almost entirely with reduced blood. Now Joffe and Poulton found that the difference between the CO_2 content of blood and that of its true plasma was slightly less, if the blood was reduced, than if it was oxygenated. This difference was about 1.7 cc. % and this has been subtracted from the plasma figures obtained from Campbell and Poulton's curve.

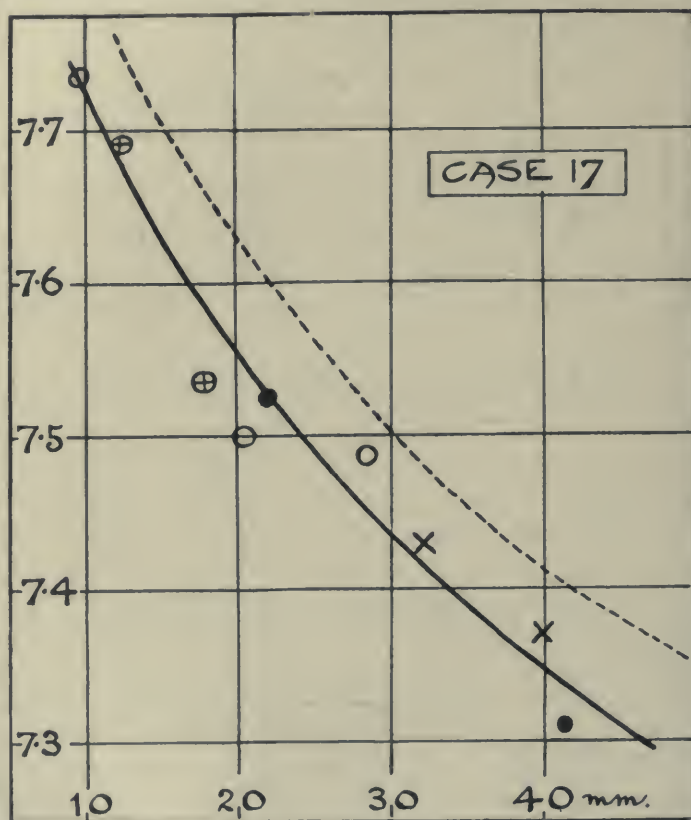


Fig. 3. The dotted line represents the normal relation between p_{H} and CO_2 tension in the reduced blood of T. R. P.

The points indicate values of this relationship in the blood of case 17, obtained as follows:

● By direct electrometric measurement on the completely reduced blood, and subtraction of 0.038 from each observed value of p_{H} , in order to bring the readings into comparison with the calculations made on the oxygenated blood.

○ By calculation from the CO_2 content of the true plasma of oxygenated oxalated blood (E. P. P.).

⊕ By calculation from the CO_2 content of the true plasma of oxygenated defibrinated blood (E. P. P.).

× By calculation from the CO_2 content of the whole oxygenated blood (E. P. P.), examined a week later.

In addition to CO_2 determinations on fully reduced blood by T. R. P. with the differential apparatus, in some cases the CO_2 was determined by E. P. P. with Van Slyke's apparatus using the technique described [Joffe and Poulton, 1920]. Sometimes fully oxygenated blood was used and sometimes partly oxygenated

blood. We have calculated the p_{H} for these results also, but where we are dealing with fully oxygenated blood we have added 0.038 to the result, which is the difference in p_{H} found by Parsons [1917] between oxygenated and reduced blood, in order to make the results comparable with the p_{H} measurements on reduced blood. When the blood was partly oxygenated a correspondingly smaller figure was added. These results of E. P. P.'s form a useful check on T. R. P.'s results especially as the methods used are quite different. On the whole the agreement is good.

We have used the same blood-true plasma CO_2 relation in calculating the p_{H} in our case of polycythaemia, because there is evidence that this relation is unchanged in polycythaemia, where the fixed acid of the blood is increased.

There is one other point. Campbell, Hunt and Poulton constructed their blood-plasma CO_2 relation for oxalated blood, while we are dealing here almost exclusively with defibrinated blood. Thus we have neglected any slight alteration there may be in the distribution of CO_2 between corpuscles and plasma, as the result of adding 0.5 % potassium oxalate.

The p_{H} values obtained by calculation and by actual experiment are given in detail in Table IV.

In Table II the differences between the values have been collected and it will be seen that out of the 57 calculations 71.9 % are not further from the observed values than 0.05. In Table I the average differences of the several determinations in each case are stated. When the fixed acid of the blood is increased, as indicated by the low value of p_{H} at 40 mm., there is a decided tendency for the p_{H} calculated from the blood-true plasma CO_2 curve to be higher than the observed value. This is shown in the last two experiments in case 4, in case 7 and in case 9. In the last case when the fixed acid in the blood due to keeping had increased greatly, the difference was 0.113, which is much the largest we found.

Table II.

Total No. of observations Percentage	No. of observations showing a difference between p_{H} (calculated) and p_{H} (observed) not greater than				Greater than 0.1
	0.01	0.03	0.05	0.1	
<i>Blood-true plasma CO₂ curve used:</i>					
57	14	26	41	53	4
%	24.6	45.6	71.9	93	
39*	13	23	31	38	1
%	33.3	59	79.5	97.4	
<i>Warburg's method used:</i>					
57	15	31	45	55	2
%	26.3	54.4	79	96.5	
39*	10	22	32	38	1
%	25.6	56.4	82.1	97.4	

* After deducting the cases in which there was a considerable increase of fixed acid, but not case 6.

On the other hand in case 3, where acid had accumulated owing to keeping, the difference was -0.048 ; but the difference was -0.06 for the same case when there was no acid, *i.e.* the alteration due to acid was in the same direction as in the other cases.

We think it possible that the relationship between the CO_2 in the corpuscles and plasma is altered by the addition of acid, so that the plasma, owing to the greater neutralisation of sodium bicarbonate contains relatively less CO_2 than the corpuscles [see Campbell, Hunt and Poulton, 1923, Section 3]. This would account for the difference. It is clear that these cases with increased fixed acid should be omitted from our test of the accuracy of the Hasselbalch formula applied to the plasma. This leaves 39 determinations and Table II shows a considerable improvement in the agreement, 79.5 % of the calculated p_{H} values not being further from the observed p_{H} than 0.05. Seven of the calculated p_{H} values coincide with the observed p_{H} , 20 are too small and 12 are too large.

Warburg¹ has recently described a modification of the Hasselbalch formula by means of which it is possible to calculate the p_{H} of blood (*i.e.* of the true plasma) from the CO_2 content of the blood and the CO_2 pressure, provided the oxygen capacity of the blood is known.

The oxygen capacities were obtained by Parsons during his log K determinations. They were carried out with various Barcroft differential apparatus in which (except for case 15) the oxygen capacity of Parsons' own blood had also been obtained. In making the calculations the value for Parsons' blood was taken at 18.5 vols. %; but it had not been determined. The haemoglobinometer value was 98 % (Price Jones).

As some of the examples in Warburg's paper are not quite accurately calculated and as the method is a valuable one, we think it best to give a fresh example of the calculation and Dr Warburg has kindly agreed to this.

Example. Case 8, on Jan. 28, 1919.

Partly oxygenated blood (% saturation = 60.3) contained 41.2 cc. CO_2 % at 41.7 mm. CO_2 pressure. It is required to find the p_{H} of this blood. The oxygen capacity (*i.e.* combined oxygen) of the blood was 17.4 vols. %.

In order to calculate the volume of free CO_2 , Warburg calculates the absorption coefficient Ψ by equation 128 and multiplies the pressure of CO_2 by the factor $\frac{0.5554}{7.6} \times \Psi$. We have shortened the process considerably by calculating a graph of the blood, which relates the oxygen capacity of the blood as abscissa with the above factor as ordinate directly. The graph is a straight line, drawn through two points such that an oxygen capacity of 1.5 vols. % corresponds to the factor 0.0709 and an oxygen capacity of 37.5 vols. corresponds to the factor 0.0620 (see Fig. 4). In the present example the factor corresponding to 17.4 vols. % oxygen is 0.0670.

¹ Dr Warburg has pointed out a mis-print to us in equation 206 [1922, p. 327] which should read $x_1 = 100 - \text{the fraction}$.

The free CO_2 is $41.7 \times 0.067 = 2.794$.

$$\begin{aligned} \text{Now } p_{\text{H}}(\text{uncorrected}) &= 6.155 + \frac{\log \text{combined } \text{CO}_2}{\log \text{free } \text{CO}_2} [\text{see Warburg, 1922, p. 202}] \\ &= 6.155 + \frac{\log (41.2 - 2.794)}{\log 2.794} \\ &= 7.293. \end{aligned}$$

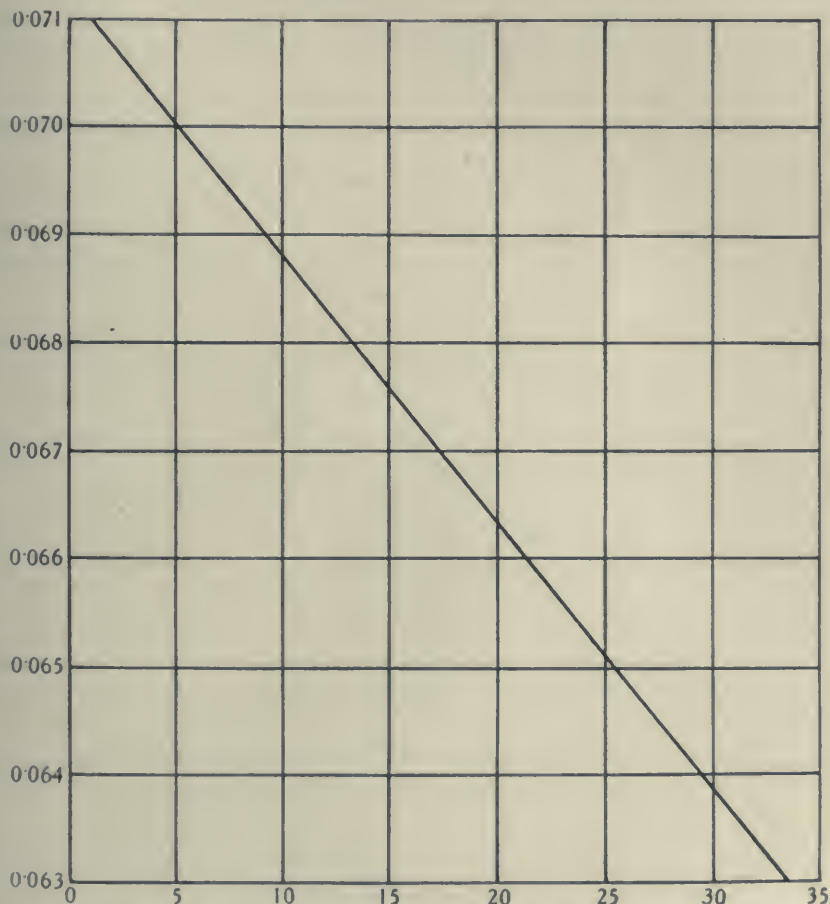


Fig. 4. Calculation of p_{H} (Warburg). Ordinate, values of F in equation—Free CO_2 (cc. per 100 cc. blood) $= F \times \text{CO}_2$ partial pressure (mm. Hg.). Abscissa, oxygen capacity of blood (cc. per 100 cc.).

The correction is obtained as follows: a value of p_{H} depending on the percentage saturation of the blood (60.3) is first calculated, viz.

$$\begin{aligned} 7.293 - \frac{100 - 60.3}{100} \times 0.40 \\ = 7.13. \end{aligned}$$

The correction corresponding to this (7.13) is obtained from Fig. 10 of Warburg's paper corresponding to 17.4 % oxygen. This is + 0.005. This is added to the original figure 7.293, so that the p_{H} is 7.298. Warburg has expressed his values

of p_H on Bjerrum's scale [see p. 167 of his paper]. Hence 0.048 must be deducted, so that the finally corrected p_H of the blood is 7.25 on the Sørensen scale. We have made an addition of 0.025 to this figure in this particular case, so as to make the result comparable with the observed p_H which was carried out on reduced blood.

We have calculated all our available results by Warburg's method and their accuracy can be compared with those calculated from the blood-true plasma CO_2 curve. A summary of the 57 results in Table II shows that the accuracy of Warburg's method is rather greater. This is because in cases 4, 7 and 9, in which the fixed acid of the blood was increased, the error is not so great by this method of calculation, as can be seen in Table I. After deducting these cases the accuracy of the two methods in the 39 remaining determinations is very much the same (see Table II). Of the 39 values calculated by Warburg's method 26 were less and 11 were greater than the observed p_H .

In this connection case 6 (erythraemia) is of special interest (Table I). On Aug. 8th after oxygen treatment the fixed acid in the blood was about normal. Under these circumstances Warburg's method of calculation would be expected to give a more accurate result, while the result calculated from the blood-true plasma CO_2 curve would be too low because with increase of blood cells there would be relatively more CO_2 in the plasma compared with the blood [see Campbell, Hunt and Poulton, 1923, Section 9]. The Warburg figure is 0.005 too large and the figure from the curve is 0.031 too small. Before treatment with oxygen the blood contained excess of fixed acid. This causes a relative diminution in the CO_2 of the plasma which neutralises the previous error in the calculation from the blood-true plasma CO_2 curve. On the other hand this makes the Warburg calculation too large. In accordance with this we find that on Aug. 3rd the former value was the more accurate, being only 0.005 too large, while the Warburg calculation was 0.013 too large.

In case 12 the oxygen capacity was rather low, without increase of fixed acid. This would make no difference to the Warburg figure but would make the figure calculated from the curve too high. In accordance with this we find that the former was only 0.002 too high, while the latter was 0.025 too high.

The conclusion we would draw is that when the haemoglobin content is about normal both methods of calculation from the CO_2 content of the blood are available; where the haemoglobin is distinctly too high or too low and there is no increase of fixed acid or base, Warburg's method is the better; where there is increase or diminution in fixed acid, it is necessary to determine the CO_2 in the true plasma and make the calculation from this by Hasselbalch's formula using a constant value of 6.096 for p_{K_1} .

4. THE p_H OF REDUCED BLOOD AT 40 MM. CO_2 PRESSURE.

The method which has been widely adopted for obtaining a measure of the fixed acid in the blood was described by Van Slyke and Cullen [1917],

viz. determining the alkali reserve of separated plasma (the CO_2 combined at 40 mm.). A better plan is to determine the CO_2 combined at 40 mm. in whole blood [Joffe and Poulton, 1920]. It is permissible in most cases to draw conclusions as to the fixed acidity of the blood from these measurements, providing that the blood has about a normal haemoglobin content. It is still better to determine the CO_2 combined in the true plasma, as this gives a measure of the amount of fixed acid present, irrespective of the haemoglobin content of the blood. Undoubtedly the best method of measuring variations in fixed acidity is to determine the reaction of the blood by some direct method at a known pressure of CO_2 such as 40 mm. For this purpose we have used the hydrogen electrode, and figures have been obtained for 17 cases, some of them being examined on several occasions.

The normal limits for the p_{H} of reduced blood at 40 mm. CO_2 pressure have been determined by Barcroft and his co-workers [1922] by observations on nine different subjects. The values ranged from 7.38 to 7.47. Parsons' blood at one time gave a value of 7.36 [Donegan and Parsons, 1919], so that we may regard 7.36–7.47 as the normal limits.

In our series of 17 cases results between normal limits were obtained in seven and six of them lay between 7.36 and 7.39, so that they were on the acid side of Barcroft and his co-workers' mean value. The seven cases comprised two each of diabetes, mitral disease, and myocardial disease, and one normal (15) in which the blood had been kept overnight. In the other normal case (5) the value was 7.34, which is slightly acid; but for some unexplained reason the fixed acidity was still further increased after treatment in an oxygen chamber.

Cases with evidence of heart failure also gave mostly normal results. These included two cases of mitral stenosis—case 12, $p_{\text{H}} = 7.37$, and case 14, $p_{\text{H}} = 7.46$, and four cases of myocardial degeneration which were clinically similar to the cases described by Lewis and his colleagues [1913]. They were: case 2, 7.335; case 11, 7.35; case 16, 7.36; and case 17, 7.36. These results agree with those of Campbell, Hunt and Poulton [1923] and indicate that there is no large amount of fixed acid present in the blood, as has been suggested.

In one case of diabetes with very little ketosis (case 3) the p_{H} was normal, 7.39. In case 13 where the ketosis was not marked on one occasion, due to treatment, the p_{H} was 7.39, though on a second occasion when ketosis had increased the value of 7.265 was obtained showing considerable increase in the fixed acid of the blood. Case 4, azotaemic nephritis, gave a value of 7.34 on one occasion, but on a second occasion three months later the value was 7.23 before and 7.22 after oxygen, showing a considerable increase in the fixed acid of the blood.

Six of our cases showed a marked increase in the fixed acidity. One of these (case 1) was a case of uraemia following septic pyelonephritis. The p_{H} of the blood while the patient was unconscious shortly before death was 6.72, the blood being actually acid, but it had been kept 16 hours longer than usual

although in ice all the time. The other was a case of parenchymatous nephritis with oedema, 7.29 and 7.295 on two occasions.

Two cases of diabetes with ketosis showed increase in the fixed acidity. In case 10 this was slight, the p_H being 7.295. In case 9 values of 7.14, 7.04 and 7.15 were obtained. The middle one of these determinations—7.04—was done when the patient was approaching coma, but the blood had been kept in ice overnight.

In one case of mitral stenosis (case 7) with pregnancy the p_H was 7.26, the acid value being probably accounted for by the pregnancy (see Hasselbalch and Gammeltoft, 1915].

In case 6 (erythraemia with splenomegaly) the p_H was 7.25, showing increase in fixed acidity. The interesting point was that after treatment with oxygen the p_H rose to 7.35 a normal figure, so that the acid, whatever it was, was abolished by oxygen. Dr Price Jones found that the condition of the blood remained unaltered (Table III). This suggests that the polycythaemia was not secondary to want of oxygen [see Weber, 1921].

In Table I the fixed CO_2 blood at 40 mm. is also given. On the whole the values diminish with the p_H , as would be expected; but there are surprising exceptions, *e.g.* case 4, where the CO_2 remained much the same, while the fixed acid, as indicated by the p_H , had increased considerably.

Table III.

Case	Date	Red cells $\times 10^6$ white cells per cu. mm.	Hb. per cent. colour index	Mean diameter of red cells	Differential Count, per cu. mm.				
					Poly- morphs	Lympho- cytes	Hyalines	Eosino- phils	Mast cells
4	24. vi. 18	5.41	86	7.44	6922	6306	1050	293	117
		14,666	0.79	—	—	—	—	—	—
	29. vi. 18	4.136	88	7.55	3793	3863	835	157	52
5	(after O_2 chamber)	8,700	1.07	—	—	—	—	—	—
	5. vi. 18	4.856	94	7.56	7330	2415	588	147	21
		10,500	0.96	—	—	—	—	—	—
	10. vi. 18	4.86	98	7.34	6271	4680	468	281	0
6	(after O_2 chamber)	11,700	1.01	—	—	—	—	—	—
	3. viii. 18	8.346	158	6.87	4350	390	150	100	10
		5,000	0.95	—	(10 normoblasts, 10 myeloblasts)	647	251	145	53
	9. viii. 18	8.646	164	6.91	5504	647	251	145	53
	(after O_2 chamber)	6,600	0.95	—	(40 normoblasts)	—	—	—	—

5. p_H OF REDUCED BLOOD AT ALVEOLAR CO_2 PRESSURE.

This calculation has often been made in order to express the p_H of the arterial blood on the assumption that the alveolar and arterial CO_2 pressures are identical. In the paper by Campbell, Hunt and Poulton [1923] it is shown that this approximately holds in people who are not breathless, and in anaemia and erythraemia, while in cases of mitral stenosis there is sometimes fairly good correspondence and sometimes not. However in breathless cases where there was evidence of pulmonary disease the alveolar samples gave results very much below the arterial CO_2 pressure. Many of these cases might be classified as cardio-renal, although there was evidence that the lungs were

also affected. On this account we have not calculated the p_{H} at alveolar CO_2 pressure for cases 2, 16, 11 and 17, as we think the results would be misleading.

The figures in column 5 (Table I) are for fully reduced blood and it is necessary to deduct about 0.04 to make these strictly comparable to the figures for arterial blood. The results, as is the case with all figures hitherto published, may err somewhat on the acid side, as the observations were made before Lovatt Evans' work on glycolysis was published. This subject is dealt with fully elsewhere [Campbell, Hunt and Poulton, 1923].

Approximately normal results were obtained in cases 5, 15 (normals) and in case 4 on March 4th while the p_{H} fell somewhat as the fixed acid in the blood was increased later and in case 8 (tubal nephritis). In case 6 (erythraemia) the p_{H} was 7.27 which is low and this was fairly close to the arterial value for case 13 of the next paper. In case 12 (mitral stenosis) the value 7.42 was quite close to the arterial value 7.395 of the same patient in Campbell, Hunt and Poulton's paper (case 9). In case 27 the value was normal showing that the increase of fixed acid due to pregnancy had been approximately compensated. In case 1 (uraemia) there was great acidemia, but the blood had been kept longer than normal. Still, taken in conjunction with case 33 of Campbell, Hunt and Poulton's paper there can be little doubt that acidemia is present in uraemic coma, but evidence that the coma is not itself due to the acidemia is given in their paper.

In the ketosis of severe diabetes there are reasons [Campbell, Hunt and Poulton, 1923] for believing that the alveolar samples may give too low values for the arterial CO_2 . If this is the case, the arterial p_{H} would probably lie somewhere between the value at alveolar CO_2 pressure and the value at 40 mm. This would suggest that there was a measurable amount of acidemia in case 9 who was verging on a state of coma when the examinations were made. Sonne and Jarlöv [1918] found the same thing by determinations of the CO_2 of blood at 40 mm. Poulton [1918] also came to this conclusion from log K determinations. However it does not follow that this acidemia is itself the cause of diabetic coma. Cases of emphysema with myocardial changes are described by Campbell, Hunt and Poulton, and in these cases there was for months some degree of acidemia due to CO_2 without any symptoms of drowsiness. However it must be admitted that in the case of diabetes further observations with analysis of the arterial blood are necessary before conclusions can be drawn as to whether acidemia plays any part in the symptomatology of coma. On other grounds this is unlikely [Poulton, 1918].

6. THE BUFFER VALUE OF BLOOD.

Following Barcroft, Bock, Hill, Parsons, Parsons and Shoji [1922] we have considered that buffer value should be expressed as a change in c_{H} and we define it as the percentage volume of CO_2 it is necessary to add to blood within the physiological range, to increase the c_{H} by 1×10^{-8} .

The buffer value of blood is difficult to estimate because if only a few p_{H}

and CO_2 points are determined, a slight error in the determination will make a considerable change in the values obtained. In many of our cases we have not only the CO_2 volumes determined on the same samples of reduced blood which were used for the p_{H} measurements, but we have also CO_2 measurements on the partly oxygenated blood used for the $\log K$ determinations. These latter CO_2 determinations have not so far been employed, and are not given in the protocols, because the constant of the differential apparatus used was not known with the same degree of accuracy as that of the apparatus used for the reduced blood. However the results are comparable among themselves, so that they can be used for obtaining the buffer value. In Table I calculations from the CO_2 of both reduced and partly reduced oxygenated blood are given and the number of points available for the calculation are put in brackets after the value.

The most striking fact arising out of these results is the effect that increasing the fixed acid of the blood has in diminishing the buffer value. Taking cases 3, 4, 7 and 9, when the p_{H} at 40 mm. was not above 7.26 the average buffer value was 4.54, while taking the cases where the p_{H} at 40 mm. was above 7.33, the average buffer value was 7.0.

Anaemia also diminishes buffer value; but this can hardly be of much importance in these cases because the average oxygen capacity was not very different in the two groups, being 17.1 in the first and 19.1 in the second.

The remarkably low value of 1.1 was obtained in case 1 (uraemia), where the fixed acid was very greatly increased, and there was also considerable anaemia.

In conclusion, we should like to thank Dr Price Jones for allowing us to quote some of his blood findings, and Dr W. N. Hurtley for kindly analysing the urine in case 13.

The expenses of this investigation were defrayed by Government Grants from the Royal Society and a maintenance grant to one of us (T. R. P.) from the Medical Research Council.

7. CONCLUSIONS.

(1) The $\log K$ - p_{H} relationship was a straight line, in most cases parallel to the P.-B. line and to the line for Parsons' blood, but it was usually closer to the latter. In cardio-renal disease and mitral disease with heart failure the $\log K$ at p_{H} 7.4 tended to be rather low. It was rather higher in a few cases of diabetes with ketosis.

(2) The p_{H} can be calculated from the CO_2 content of blood and the CO_2 partial pressure, (a) by calculating the "true" plasma CO_2 from the blood—true plasma CO_2 curve given by Campbell, Hunt and Poulton [1923], (b) by Warburg's method. The latter method must be used where there is any great increase or diminution in the oxygen capacity of the blood. If there is increase or diminution of CO_2 fixing power, it is essential to determine the CO_2 content of the true plasma and use this for the calculation. In suitable cases 80 % of values were found not to be further away from the observed p_{H} than 0.05.

(3) A graph has been constructed which should be useful in calculating p_{H} values by Warburg's method.

(4) A case of erythraemia with splenomegaly, treated for five days in an oxygen chamber, at Cambridge, showed no alteration in the blood picture. There was a marked diminution in the fixed acid of the blood, which was previously abnormally high.

(5) In cases of diabetes with ketosis, in mitral disease with pregnancy, in uraemia and renal disease and erythraemia, the p_{H} observed at 40 mm. was abnormally low. In other cases normal values were usually obtained.

(6) The buffer value of blood is diminished, as the fixed acid in blood is increased.

8. CLINICAL NOTES ON CASES AND PROTOCOLS.

Case 1. *Male*. Enlarged prostate. Suppurative pyelo-nephritis. Uraemia. Blood urea 0.4 %.

Case 2. *Male*. Myocardial degeneration, nephritis and bronchitis, anasarca, blood urea 0.046 %.

Case 3. *Female*. Diabetes mellitus. Faint Rothera reaction.

Case 4. *Male*. Azotaemic nephritis following streptococcal pneumonia. Blood urea on Oct. 26, Nov. 10, 1917, Feb. 7, March 15, June 12, 1918, respectively 0.2, 0.09, 0.08, 0.05, 0.07 %. B.P. (Mar. 19) 183/110. Uraemia and death in March 1921. Blood urea 0.44 %.

Case 5. *Healthy Male*. Recovery after having suffered in Jan. 1918 from acute nephritis and uraemia with hemiplegia. Blood urea on Jan. 29, Feb. 12, April 10, 25, May 7, respectively 0.23, 0.03, 0.03, 0.03, 0.02 %.

Case 6. *Male*. Erythraemia with enlarged spleen. B.P. 160.

Case 7. *Female*. Mitral stenosis, 4-5 months pregnancy. Some bronchitis.

Case 8. *Male*. Chronic tubal nephritis, lipaemia, oedema. Blood urea 0.02 %.

Case 9. *Male*. Diabetes, ketosis, lipaemia, sepsis. Ammonia index 23.3 %. Coma threatening on Feb. 3 with subsequent recovery. Died a few weeks later.

Case 10. *Male*. Diabetes, ketosis, septic glands.

Case 11. *Male*. Myocardial degeneration, emphysema, pulsus alternans, anasarca, oedema of lungs, albuminuria. Blood urea 0.04 %. Cheyne-Stokes respiration. No cyanosis. Large tender liver.

Case 12. *Female*. Mitral stenosis and regurgitation. Bronchitis. Oedema. Large liver. No cyanosis. (The same as case 9 of Campbell, Hunt and Poulton's paper.)

Case 13. *Female*. Diabetes, ketosis. Given 3 drachms sod. bicarb. daily. June 2, Urine sugar 3.6 %; Ammonia index 8 %; Aceto-acetic acid slight. Oct. 21, ketosis had much increased. Dr W. N. Hurtley of St Bartholomew's Hospital very kindly analysed the urine (184 cc.) collected during the 12 hours, 8 p.m. to 8 a.m., before the blood sample was taken. Aceto-acetic acid was

0.19 %; total 3.51 gm. The β -hydroxybutyric acid was 0.63 %; total 11.6 gm. as determined by rotation and 0.94 %; total 17.3 gm. as determined by titration. The ratio $\frac{\beta\text{-hydroxybutyric acid}}{\text{total acetone bodies}}$, was 82.6 % agreeing with Neubauer's and Kennaway's findings [1914] in severe diabetes.

Case 14. *Female*. Mitral stenosis and regurgitation, large pulsating liver, ascites, right sided dilatation of heart, lips slightly cyanosed, orthopnoea.

Case 15. *Male*. Tabes with myatroph, taken as a normal control.

Case 16. *Male*. Fibroid heart (650 g.), Atheroma of coronary arteries, Infarcts of spleen and kidneys. Auricular fibrillation. Oedema, periodic breathing and orthopnoea. Distinct cyanosis of face and hands. Blood urea 0.04 %. Slight albuminuria.

Case 17. The same as case 26 [Campbell, Hunt and Poulton, 1923].

Table IV.

Case	Date	Fully reduced blood				Partly oxygenated blood	
		CO ₂ mm.	p_{H} (observed)	p_{H} (calculated)		CO ₂ mm.	log K
				Bl. t. pl. curve	Warburg		
1.		9.9	6.92	—	—	—	—
		26.0	6.81	—	—	10.5 ¹	5.74
		40.2	6.72	—	—	23.7 ¹	5.66
2.		23.1	7.51	7.51	7.49	39.4	4.43
		33.1	7.39	7.39	7.38	44.2	4.33
		41.7	7.33	7.33	7.315	49.0	4.25
3. Feb. 27		27.1	7.40	7.34	7.33	31.8	4.32
		35.4	7.30	7.265	7.25	35.5	—
Mar. 4		44.3	7.37	7.29	7.29	48.1	—
4. Mar. 13		27.3	7.465	7.39	7.40	36.7	30.4
		48.9	7.27	7.24	7.24	47.0	56.4
June 24		2.5	7.84	—	—	8.2	11.6
		25.0	7.34	7.435	7.42	36.2	35.7
		40.8	7.23	7.265	7.24	40.6	49.2
		61.6	7.11	7.15	7.13	48.8	63.3
June 29		2.3	7.96	—	—	5.0	0.5
		23.5	7.37	7.42	7.405	33.0	18.6
		42.7	7.20	7.31	7.30	47.9	32.9
		64.9	7.07	—	—	—	64.5
5. June 5		1.7	8.17	—	—	11.8	11.8
		16.8	7.61	7.555	7.54	30.9	26.9
		45.6	7.32	7.29	7.285	49.4	38.8
		33.2	7.39	7.375	7.37	42.7	52.4
June 10		1.9	8.18	—	—	5.6	10.2
		17.9	7.56	7.60	7.605	38.0	20.8
		31.0	7.37	7.41	7.395	42.4	41.1
		45.3	7.24	7.285	7.28	48.4	57.1
6. Aug. 3		58.9	7.17	7.20	7.175	50.7	55.7
		22.2	7.36	—	—	—	—
		40.6	7.24	—	—	—	—
		60.4	7.13	—	—	—	—
Aug. 8		12.5	7.63	—	—	—	—
		38.8	7.36	—	—	—	—
		60.7	7.23	—	—	—	—
		35.0	7.29	7.335	7.315	40.8	31.6
7.		49.8	7.20	7.205	7.19	44.7	45.8
		30.0	7.38	7.37	7.375	39.3	24.0
8. Dec. 18		39.6	7.30	—	—	—	38.8
		—	—	—	—	—	50.3
Jan. 28		37.5	7.32	7.35	7.34	45.4	16.7
		—	—	—	—	—	30.3
		—	—	—	—	—	40.0

Table IV *continued*.

		Fully reduced blood				Partly oxygenated blood	
		p_{H} (calculated)					
Case	Date	CO ₂ mm.	p_{H} (observed)	Bl. t. pl. curve	Warburg	CO ₂ cc. %	CO ₂ cm. log K
9.	Jan. 14	16.0	7.33	7.405	7.36	20.3	16.1 4.26
		29.3	7.22	7.24	7.205	27.0	31.4 4.13
		49.1	7.08	7.105	7.07	34.5	— —
Feb. 4		15.1	7.245 ²	7.425	7.355	18.8	14.0 4.496
		14.6	7.25	—	—	—	29.4 4.14
		29.2	7.105	7.15	7.065	20.1	— —
Feb. 11		9.4	7.44	7.555	7.525	16.8	11.2 4.27
		26.8	7.22	7.26	7.225	25.5	39.2 4.20
		36.3	7.17	7.235	7.205	33.6	— —
10.		20.3	7.465	7.465	7.455	31.0	22.1 4.47
		42.9	7.28	7.23	7.215	40.3	35.4 4.40
		—	—	—	—	—	43.4 4.29
11.		16.3	7.59	7.59	7.58	32.8	16.2 4.16
		41.2	7.345	7.33	7.31	47.5	37.2 4.09
12.		19.8	7.545	7.59	7.565	39.8	19.5 4.58
		28.3	7.45	7.48	7.45	44.4	29.5 4.02
		41.7	7.37	7.37	7.355	53.8	46.7 4.17
13. June 2		20.5	7.62	—	—	—	— —
		28.9	7.52	—	—	—	— —
		44.3	7.34	—	—	(Oct. 21)	11.7 4.80
Oct. 21		20.9	7.45	—	—	—	26.0 4.64
		39.6	7.27	—	—	—	35.5 4.57
		27.8	7.575	—	—	—	29.9 4.41
14.		40.4	7.46	—	—	—	— —
		29.3	7.46	7.325	7.30 ³	32.8	28.2 4.37
15.		49.8	7.30	7.26	7.255 ³	50.1	47.7 4.29
		20.7	7.565	7.495	7.515	34.4	21.7 4.49
16.		41.7	7.35	7.29	7.30	44.8	41.0 4.30
		21.5	7.56	—	—	—	— —
		41.5	7.35	—	—	—	— —
Experiments on fully oxygenated blood							
4. Mar. 13		29.8	7.43 ⁴	7.415	7.42	36.5	— —
		38.4	7.35 ⁴	7.385	7.365	42.3	— —
13. Oct. 21		21.0	7.415 ⁵	—	—	—	— —
		39.3	7.24 ⁵	—	—	—	— —
16. Mar. 13		20.7	7.51 ⁵	—	—	—	— —
Experiments on partly oxygenated blood (Hb. circ. 50 % saturation)							
6. Aug. 3		16.0	7.41 ⁶	7.41 ⁷	7.405 ⁷	19.1	— 4.39
		37.2	7.26 ⁶	7.265 ⁷	7.28 ⁷	34.5	— 4.12
		53.3	7.17 ⁶	7.18 ⁷	7.195 ⁷	42.7	— 5.98
Aug. 8		9.9	7.66 ⁶	7.665 ⁷	7.705 ⁷	21.4	— 4.50
		27.9	7.455 ⁶	7.385 ⁷	7.415 ⁷	33.8	— 4.36
		43.1	7.335 ⁶	7.305 ⁷	7.34 ⁷	45.0	— 4.17
8. Jan. 28, '19		58.4	7.24 ⁶	7.21 ⁷	7.24 ⁷	50.0	— 4.09
		15.4	7.53 ⁶	7.475 ⁷	7.435 ⁷	21.9	— —
		41.7	7.285 ⁶	7.29 ⁷	7.275 ⁷	41.2	— —
9. Jan. 14, '19		32.6	7.19 ⁶	7.245 ⁷	7.20 ⁷	28.6	— —
		14.8	7.53 ⁶	7.48 ⁷	7.435 ⁷	20.6	— 4.66
10.		35.7	7.32 ⁶	7.295 ⁷	7.28 ⁷	35.9	— 4.327

¹ Corresponding values for CO₂ content were 5.9 and 8.6 cc. %.² By extrapolation.³ Assuming haemoglobin content of blood was normal.⁴ Values obtained by interpolation from reduced blood, with 0.038 added, so that they represent p_{H} of fully oxygenated blood [see Parsons, 1917].⁵ Oxygenated true plasma was used for these determinations.⁶ These values are for reduced blood, obtained by interpolation from the values given earlier in the table.⁷ CO₂ in partly oxygenated blood was used in these calculations, which were subsequently corrected so that they represent values of p_{H} for reduced blood.

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XXXIX. THE EFFECT OF A YEAST EXTRACT ON THE OXYGEN CONSUMPTION OF WASHED FROG MUSCLE.

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IN 1911 Batelli and Stern [1911] showed that if some finely divided mammalian muscle was either allowed to respire until the velocity of its respiration was somewhat diminished or was washed with a little cold water, which produced a similar effect, the addition of an extract of various mammalian tissues made with cold water increased the velocity of the respiration and in some cases restored it to its initial value.

They showed that the effect was temporary, but that a second addition of such a tissue extract produced a second, temporary, increase in the velocity of respiration. They assumed that a substance which they called "Pnein" was present in these extracts. They found that by a short period, 45 minutes, of respiration, the "Pnein" was not destroyed to any great extent.

In 1918 and 1919 Meyerhof working on "acetone-yeast" [1918] and frog muscle [1919, 1] showed that in the case of each of these substances the velocity of respiration could be very considerably reduced by exhaustive washing and could afterwards be restored by the addition of an extract of yeast or of animal tissues made with boiling water ("Koch-saft").

He assumed the presence in these extracts of a substance or a mixture of substances, which accelerated the velocity of respiration, and named it the "Respiration-substance" (Atmungs-körper). He advanced the hypothesis that the "respiration-substance" probably consists of a "co-enzyme to respiration" which is an oxygen-carrier [Meyerhof, 1919, 2] and is identical with the co-enzyme to alcoholic fermentation [Harden and Young, 1906], together with a supply of oxidisable material. He observed that, on warming such an extract for some hours in the air [Meyerhof 1918], a portion of the "respiration-substance" was destroyed.

The experiments described in this paper were undertaken in an attempt to obtain evidence as to the actual existence of a "co-enzyme to respiration" of the type suggested by Meyerhof.

Some finely chopped frog's muscle was washed several times with distilled water. It was then aerated for several hours at the ordinary temperature of

the laboratory whilst suspended in a yeast "Koch-saft." The liquid was then filtered off from the muscle, and used as the suspension fluid in which more freshly washed frog's muscle was allowed to respire in a Barcroft micro-respirometer, by means of which its oxygen uptake was measured. Some more of the same "Koch-saft" was stored at 0° out of contact with the air for the same period of time and its effect on the respiration of washed frog's muscle was compared with that of the aerated "Koch-saft."

If the "respiration-substance" were a true co-enzyme and the oxidisable substances were contained in the washed muscle the velocity of respiration in the two cases should not differ considerably. If, however, the oxidisable substances were contained in the "Koch-saft" the unused one should be the more active. In the latter case the existence of a respiration co-enzyme would not be disproved. The assumption of its existence would, however, seem to be unnecessary.

EXPERIMENTAL DETAILS.

The yeast extracts were made by boiling some baker's yeast with about twice its weight of distilled water for a few minutes and then filtering as quickly as possible. They were adjusted with caustic soda to p_H 7.6, and divided into two portions. One was passed through a Berkefeld filter and stored in small evacuated tubes in an ice-chest. The other portion was aerated at room temperature in contact with some frog's muscle which had been washed several times with distilled water. The exact figures for each case are given in the table of results. It was then filtered from the muscle, passed through a Berkefeld filter and stored in an ice-chest until it was used. In order to determine the effects of the two portions on the respiration of washed frog's muscle some more of the latter was taken and half a gram weighed out into each of three respirometers. In them were placed 3 cc. respectively of each of the two samples of "Koch-saft" and of a 1 % solution of KH_2PO_4 , each being adjusted with caustic soda to p_H 7.6. They were placed in a bath maintained at a known temperature and shaken by means of an electric motor. Soda was placed in the cups to absorb the carbon dioxide evolved.

In experiments 7 and 8, to the yeast extract was added an excess of saturated lead acetate solution and enough of a suspension of lead hydroxide to keep the liquid slightly alkaline. This removes a large number of substances, among them glutathione and any inorganic phosphates. The precipitate was filtered off and the excess lead was removed from the filtrate with sulphuretted hydrogen and the solution concentrated *in vacuo*. The acid potassium phosphate was added in experiment 8 in accordance with the discovery by Meyerhof that it makes the respiration velocity more nearly constant during the experiment.

It can be seen from the table of results that during respiration a great part of the "respiration-substance" is destroyed.

		1st respiration					2nd respiration					Frog's muscle	
							cmm. O ₂ absorbed per gram of muscle in one hour						
No.	Extract	Vol. cc.	Wt. of frog's muscle grams	Times washed	Vol. of H ₂ O each washing	Time of aeration hours	Temp. °C.	(1)	(2)	(3)	Times washed	Vol. of H ₂ O each washing	
								In phosphate	In previously respired extract	In stored extract			
1	Yeast	30	2.5	6	50	18	23	36	98	424	7	50	
2	"	45	4.5	8	50	18	22	42	88	254	8	75	
3	"	30	3	6	60	18	23	20	20	296	8	50	
4	"	26	2.6	8	50	21	22	30	33	296	8	50	
5	"	50	5	6	50	23	22	33	147	420	8	80	
6	"	50	5	6	50	45	21.5	37	116	337	8	80	
7	Yeast filtrate after basic lead acetate	50	5	6	50	44	23.5	43	58	558	8	60	
8	As 7 but with 1 % KH ₂ PO ₄ added	40	4	6	50	20	21.5	28	105	449	8	75	

DISCUSSION OF RESULTS.

Meyerhof observed that the addition of yeast or of tissue extracts to washed tissue increases the velocity of its respiration. He attributed this effect to the presence in such extracts of a co-enzyme which, working in conjunction with the washed tissue, increased the velocity of the oxidation of the oxidisable substances present. It seemed to him that the known oxidisable substances were present in too small a concentration to account for the effects observed.

Research, subsequent to the publication of Meyerhof's papers, has extended our knowledge of the number and concentration of oxidisable substances contained in such extracts. This knowledge, in conjunction with the results described in this paper, suggests that the stimulation of the respiration of washed tissues, observed on the addition of such extracts, may reasonably be attributed to the united effects of the different oxidisable substances therein contained, rather than to the presence of a co-enzyme, the necessity for which whether as an oxygen-carrier or in any other capacity has not yet been clearly demonstrated.

SUMMARY.

If an extract of yeast, made with boiling water, be aerated for some hours at room temperature in contact with some washed frog's muscle it loses its power to increase the velocity of oxygen uptake of another preparation of fresh washed muscle. This suggests that the effects attributed to a "respiration-substance" capable of providing a general stimulus to intracellular oxidative processes are really to be attributed to the collection of oxidisable substances contained in the yeast extract. When these have been oxidised the extract has no further power to stimulate the respiration of washed frog's muscle.

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XLX. THE EFFECT OF AIR, WHICH HAS BEEN EXPOSED TO THE RADIATIONS OF THE MERCURY-VAPOUR QUARTZ LAMP, IN PROMOTING THE GROWTH OF RATS, FED ON A DIET DEFICIENT IN FAT-SOLUBLE VITAMINS.

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Introductory.

It has already been demonstrated [Hume, 1922; see also Goldblatt and Soames, 1922] that young rats fed on a diet deficient in fat-soluble vitamins, grow normally for a much longer period, when they are irradiated with the mercury-vapour quartz lamp, than when not so treated. Experiments by Kestner [1921, 1922], to which attention was directed by Professor Hans Meyer, Vienna, suggested the possibility that exposure to a source of ultra-violet light might not be the only means of thus inducing growth, but that exposure to air through which such light had passed might also be effective. Kestner sought to disentangle the factors which give to a mountain climate its well-known health promoting properties. It had already been observed [Laquer, 1913, 1919; Weber, 1919] that in dogs rendered anaemic, the red blood corpuscles are regenerated more quickly at an altitude (Monte Rosa) than in the plains, an effect generally attributed to the reduced oxygen pressure at high altitudes. Kestner made his tests with dogs rendered anaemic by bleeding or by injection of pyrocin, and he observed the rate of regeneration of red cells in control animals and in those under the influence of (a) reduced atmospheric pressure, (b) the light from a naked carbon arc lamp, and (c) the air drawn from the neighbourhood of such a lamp. Reduced pressure produced no more rapid regeneration than occurred in control animals, but the direct rays of the lamp or air drawn from the neighbourhood of the lighted lamp both caused the regeneration to be more rapid and complete. It seemed therefore worth while to test whether air which had been

exposed to a source of ultra-violet light, also possessed the power of prolonging normal growth in rats fed on a diet deficient in fat-soluble vitamins. This was tested by means of the following experiments. Experiment 1 was performed in Vienna and experiments 2-4 at the Lister Institute.

The diet deficient in fat-soluble vitamins had the following composition: heated caseinogen, 180 g.; maize starch, 520 g.; hardened cotton-seed oil, 150 g.; salt mixture, 50 g.; marmite (yeast extract), 60 g.; lemon-juice, 50 g.; water, 600 g. The salt mixture had the following composition: sodium chloride, 51.9 g.; mag. sulph., 164.0 g.; sodium acid phosph., 104.1 g.; potassium phosph. 286.2 g.; calcium phosph., 162.0 g.; calc. lact., 390.0 g.; ferric citrate, 35.4 g.

All rats, both treated and controls, were placed upon the deficient diet at a weight of about 50 g., and treatment, where such was instituted, began from that time or a few days later. Wherever possible controls were taken from the same litter.

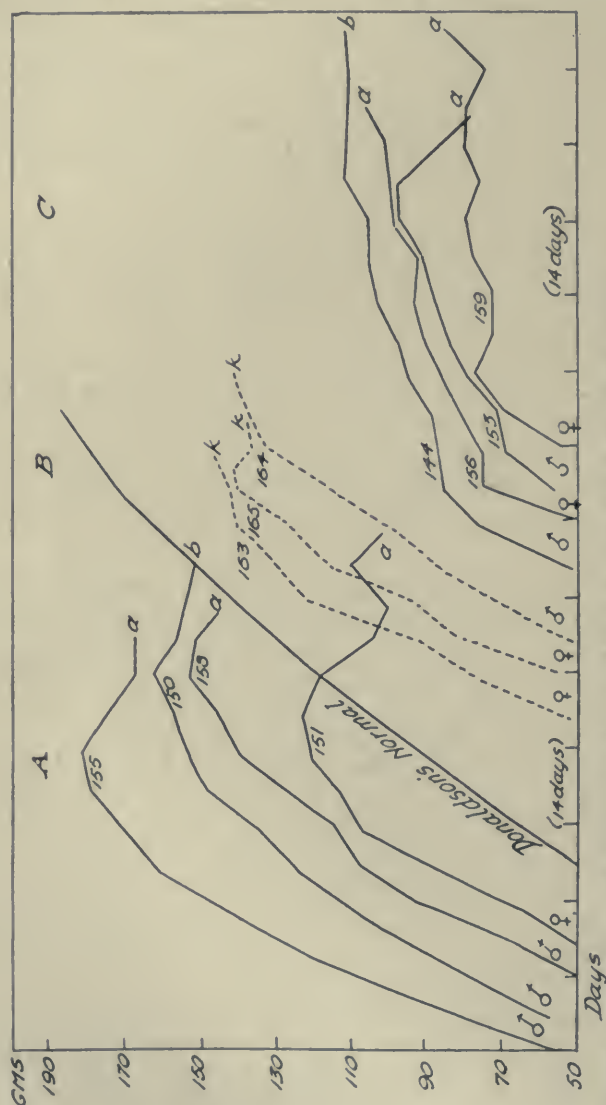
The mercury-vapour quartz lamp used for the Vienna experiments was of the Hanau pattern; it was always allowed to run for at least five minutes before being used. No estimate was made of its deterioration since purchase, but this was probably great. The lamp used for the Lister Institute experiments was a 74 pistolette quartz lamp made by the Hewittic Electric Company, using a current of 3.5 ampères and emitting rays whose shortest wave length was 230μ .

The treated rats and their controls were kept, each animal separately, in 8-litre glass cylinders, with sawdust at the bottom. The treatment with irradiated air was carried out as follows. Every second day, the rats were removed from the glass jars and left in the animal room, while the jars were conveyed to the room containing the mercury-vapour lamp, which in the Kinder Klinik was at the extreme other end of the building, and, in the Lister Institute, on another floor. The jars were placed below the lamp, with their bases about 80 centimetres distant from it, so that the light shone straight down into them. They remained in this position for ten minutes, were then covered with glass lids and conveyed back to the animal room. The animals were put in and the covers replaced to hinder diffusion of the air. *After ten minutes* the covers were removed and not replaced but the animals continued to live in the jars until the next treatment.

Experiment 1. Three animals, all from the same litter of three, were treated with irradiated air. One had been for seven days and the other two for four days, on the deficient diet, before treatment began. The weight curves, however had not begun to flatten off and even if they had done so, previous experiment [Hume, 1922] has shown that for a considerable time after being placed on the deficient diet, rats are capable of responding to the stimulus of radiation and resuming normal growth.

Control animals from the same litter were not available but other rats bred in the same way and placed upon the deficient diet a little previously,

were used for that purpose. These animals were also used as controls for a group of rats, irradiated directly with the mercury-vapour lamp, of which the details have already been published [Hume, 1922, Chart 1]. The curves of both these groups are reproduced here for comparison.



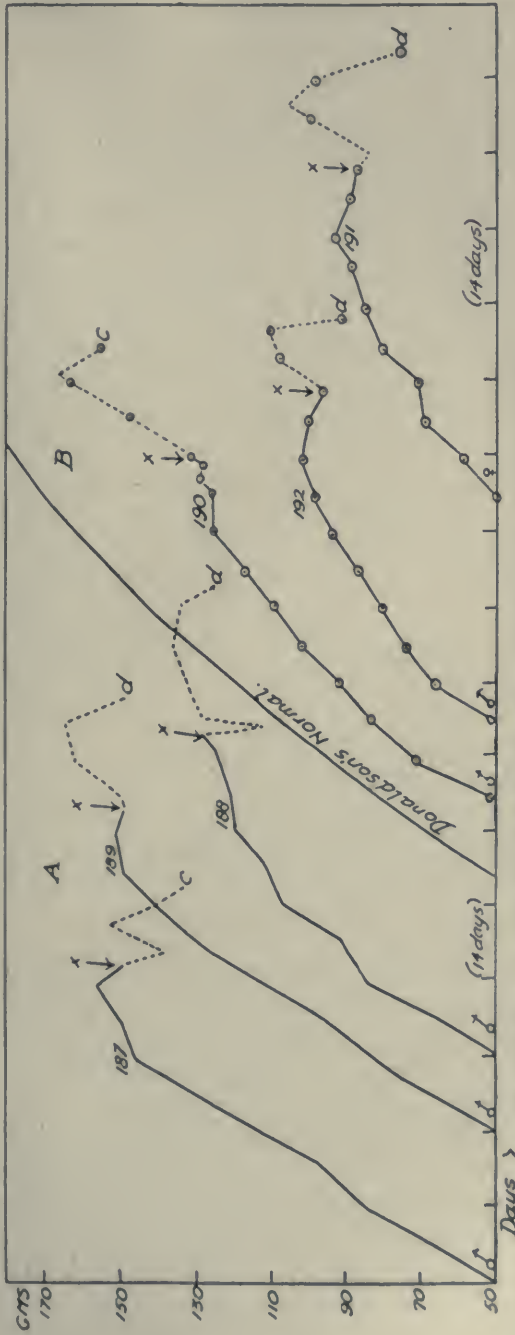


Chart 2. Experiment 2. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.

A. Treated with air, exposed to the mercury-vapour quartz lamp in glass jars for ten minutes every second day.
B. Untreated controls.
From X, the animals exposed directly to the rays of the lamp, for ten minutes daily.
(Members of the same litter are designated by the same small letter.)

as good as that of the directly radiated animals and is much better than that of the untreated controls. In the controls normal growth continued for a week, in the rats treated with irradiated air it continued for four weeks, and in the animals directly irradiated for from 3-7 weeks.

Experiment 2, Chart 2. This experiment was similar to experiment 1. Two rats from one litter, and four rats from another litter were divided into two groups of three, so that the two litters were equally represented in each group. One group of three, Chart 2 (A), was treated with irradiated air, and the other group of three served as controls, Chart 2 (B). The same superiority of the treated rats (A) over the untreated controls (B) is apparent, as in experiment 1. The controls in this case are slightly more strongly growing and the growth of the strongest control is as good as that of the weakest treated rat. Reference to the previous papers of Hume and of Goldblatt and Soames, shows, however, that this may also occur when rats are irradiated directly.

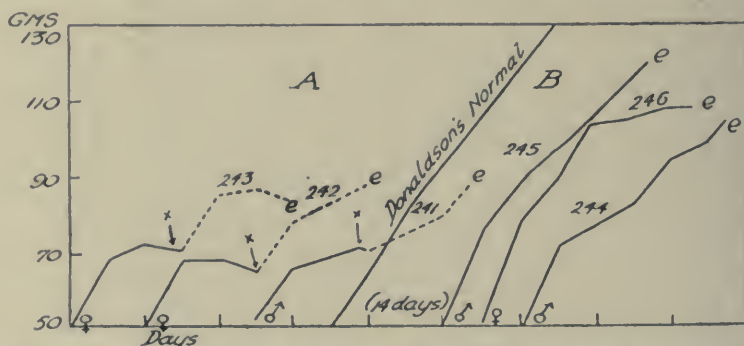


Chart 3. *Experiment 3.* Weight curves of rats fed on a diet deficient in fat-soluble vitamins.

- A. Treated for one hour daily, with air drawn from the neighbourhood of the mercury-vapour quartz lamp. From X, treatment changed, animals treated with irradiated air in jars.
- B. Untreated controls.

(Members of the same litter are designated by the same small letter.)

On the 60th day of experiment, when growth had practically ceased in every case all the six rats were exposed directly to the rays of the lamp for ten minutes every day. All made a small response and resumed growth for a period, which, except in the case of one control, was only of very short duration. The response of the controls was however the greater.

A dose of freshly irradiated air, given every second day, did not therefore evoke quite the maximum growth response, capable of being produced by direct irradiation of the animal, but it did produce a very marked prolongation of normal growth, beyond that shown by controls. A dose of irradiated air, given more often than once every second day, might be able to produce the maximum response.

Experiment 3, Chart 3. In this case the rats were exposed to air, drawn from the neighbourhood of the lighted mercury-vapour lamp, the animals being shielded from the illumination from the lamp.

A 4.5 ampère Hanau lamp was used. The lamp was surrounded by an iron shield. This iron shield which was earthed by a wire to the gas pipe was fairly air-tight, except for the ventilation holes above and below and the opening in front for the illumination to pass. The upper ventilation holes were closed with corks and a tinned funnel (10 cm. diameter) was soldered over the illumination aperture. To the stem of the funnel, 11 feet (3.3 metres) of glass tubing of 0.8 cm. outer bore, was attached, the other end of which passed through a light-tight box. The lamp was kept cool by a wet bandage around the shield.

The rats were placed singly in large bottles enclosed in a light-proof box. The bottles were closed with rubber bungs, admitting inlet and outlet tubes. Air was sucked through the whole system by a water pump. The ventilation was at the rate of 20 litres per hour. Ozone was produced in the neighbourhood of the lamp and could be detected in the bottles and at the outlet; the amount diminished as the distance from the lamp increased. After the lamp had been lit for 15 minutes, the box containing bottles and rats was brought into the room, joined up and left there for one hour daily.

Six rats were used for the experiment, all belonging to the same litter. Three were exposed to the air from the lamp and three were left untreated and used as controls.

Chart 3 shows the weight curves of the three treated rats (*A*) and of the three controls (*B*). After 20 days it was apparent that normal growth would not be prolonged by treatment with irradiated air in this way; the growth of the treated rats was inferior even to that of the controls, which in this instance showed remarkably strong growth. On the 20th day treatment with air drawn from the lamp was stopped, and treatment in jars of radiated air, as described under experiment 1, was instituted. The jars were freshly irradiated every day for ten minutes. This treatment was continued for 20 days and a moderate growth response was obtained (see Chart 3 (*A*), curves from X onwards).

It was therefore concluded that air drawn from the neighbourhood of the lamp, by means of such an apparatus as that described, does not possess the power of prolonging normal growth in rats, fed on a diet deficient in fat-soluble vitamins. The inferiority in the growth of the rats treated, to that of the controls, suggests that some factor, actually detrimental, was at work; this may have been the ozone. At any rate it seems safe to conclude that the ozone is not the growth stimulating factor.

Experiment 4, Chart 4. This experiment was a repetition of Nos. 1 and 2. In addition an extra series of jars was irradiated in the same way, but the air was blown out and fresh, not irradiated, air was admitted before the rats were placed in the jars. The jars were blown out with 20 strokes of a bellows, a process occupying a very short time, and only delaying the entrance of the rats into the jars by about 40 seconds. All jars were irradiated every day, instead of every second day as in experiment 1. Four litters of rats were used

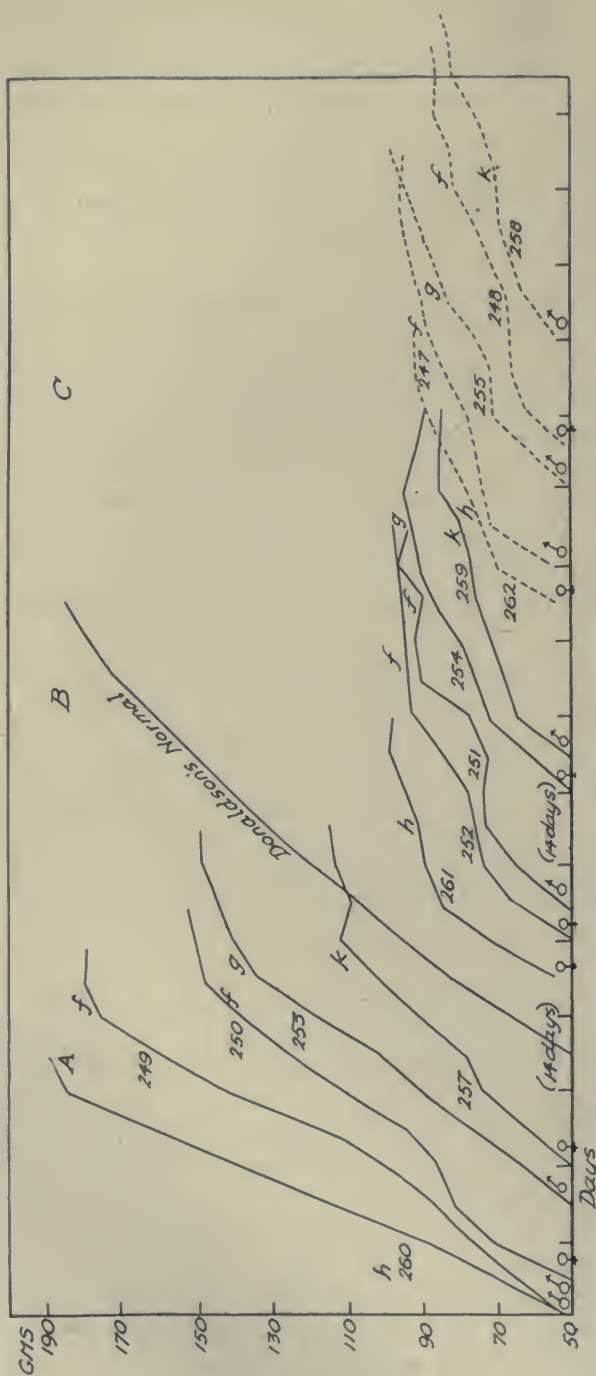


Chart 4. Experiment 4. Weight curves of rats fed on a diet deficient in fat-soluble vitamins.

A. Treated in irradiated jars containing irradiated air.

B. Treated in irradiated jars containing air not irradiated.

C. Untreated controls.

(Members of the same litter are designated by the same small letter.)

and each litter was divided equally between the three experimental groups, i.e. (A) treatment in irradiated jars, containing irradiated air, (B) treatment in irradiated jars containing air, not irradiated, and (C) control animals undergoing no treatment. Each group contained five animals. Chart 4 shows the weight curves of the three groups. The result is quite unequivocal; the rats treated with irradiated air (A) show prolongation of normal growth, while those occupying irradiated jars from which the irradiated air has been displaced, (B) behave like the untreated controls (C).

It is therefore evident that the growth promoting property lies in the irradiated air itself, and not in any property which might have been acquired by the glass.

SUMMARY.

(1) Rats fed on a diet deficient in fat-soluble vitamins, when kept in glass jars which had been exposed to the mercury-vapour quartz lamp for ten minutes every second day, showed prolongation of normal growth over controls not so treated.

(2) Rats exposed to the air containing ozone drawn over the quartz lamps and passed through 3 metres of glass tubing, 0.8 cm. bore, showed poorer growth than the control animals. The same rats, subsequently treated in glass jars of irradiated air gave some growth response.

(3) Rats placed in irradiated jars from which the irradiated air had been displaced, showed no prolongation of normal growth.

These results are analogous to the accelerated regeneration of red blood corpuscles in anaemic dogs observed by Kestner. In both cases the effect was obtained by exposure of the animals to air through which ultra-violet light has passed, as well as by exposure to the light itself. Kestner used a 40 ampère naked carbon arc lamp and the air was drawn from the neighbourhood of the lamp and passed through a box in an adjoining room in which the dogs were placed. The animals were supplied with this air for 3 to 5 hours daily.

Kestner concludes that as a result of sun radiation or radiation from the carbon arc lamp, substances are formed which, if breathed, stimulate the formation of blood corpuscles. He further suggests the possibility that compounds of nitric oxide, formed when the lamp is alight, are responsible for the action.

In the present experiments, a mercury-vapour lamp enclosed in quartz was used. This is not believed to produce nitric oxide. In experiment 3 the air drawn away from the lamp was found to possess no growth-promoting activity, but it may have been exposed for too short a time to the lamp or the character of the apparatus used may have been such as to deprive it of this activity before it could reach the rats. The negative result obtained in experiment 4 when the irradiated air in the jars was displaced makes it plain

that it is *the air and not some property impressed on the glass jars which is active*.

Ozone was present in the air in experiment 3 which gave a negative result; it is therefore clear that ozone is not responsible.

The other effect produced on air by ultra-violet radiation is a small degree of ionisation, a change which disappears rapidly. Air ionised in capacious jars, by reason of the large volume and less relative surface exposed to glass, would remain ionised somewhat longer than when drawn through glass tubes, with rubber junctions. This may explain the positive result obtained with the former method and the negative one obtained with the latter.

The authors wish to register their particular thanks to Professor Hans Horst Meyer, Pharmakologisches Institut, Vienna, for his stimulating interest and for directing attention to the work of Kestner, to Professor C. J. Martin for much help and advice; and to Dr R. Wagner for much assistance.

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XLI. THE RELATION OF CERTAIN BLOOD CONSTITUENTS TO A DEFICIENT DIET.

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(Received April 2nd, 1923.)

THE purpose of this investigation was to determine the variation of certain blood constituents with the development of polyneuritis in chickens, a subject on which, as far as we know, no work has been done.

Mature Plymouth rock hens were used for the experiment. They were given a normal ration for a short time to get them as near as possible to a normal condition. They were then fed on boiled polished rice, with filter paper added to give bulk, and crushed marble for grits.

In addition to the loss of appetite and the decrease in body weight observed by Anderson and Kulp [1922] the hens developed a craving for other foods, and would eat straw and peck at the clothing of the attendants. They were laying at first, but as the experiment progressed, there was a gradual decrease in the number of eggs produced until finally they stopped laying. The eggs had a "puckery" taste and the yolks became light coloured and smaller. Feathers did not develop where they had been plucked, when the blood was drawn. As the rice feeding continued there was a drop in temperature from 1 to 2° varying among the different chickens.

The blood was drawn from the brachial artery under the wing with a hypodermic needle, and was collected in a graduated cylinder. The needle and graduated cylinder were previously rinsed with kerosene, and potassium oxalate sufficient to prevent clotting was added. 10 cc. of blood was drawn at a time, the loss of which apparently did not affect the chickens.

Chicken blood clots more quickly than human or ox blood. There is also a wide variation among individual chickens in the tendency to clot. With some it was always difficult to secure samples; with others it was very easy. Toward the end of the rice feeding period, it became increasingly difficult in every case; and as the polyneuritis symptoms developed, it became impossible to obtain samples. The number of punctures made during the drawing of a single sample increases the tendency to clot very markedly.

Determinations of sugar, uric acid, creatinine and total non-protein nitrogen were made, using the Folin-Wu method [1919] of blood analysis (see Table I). Ogata and associates [1921] found an abnormal amount of sugar in

the blood of pigeons suffering from polyneuritis. An examination of Table I shows that in some cases the final value for sugar was greater than normal. In one individual there was a regular increase in the amount of sugar. The non-protein nitrogen values were somewhat erratic. The uric acid and the creatinine values showed the most regular variation, decreasing at first then suddenly increasing. Soon after this increase occurred, the hen showed signs of polyneuritis. The increased uric acid was apparent from two to five weeks before paralysis set in. Autopsies of the chickens which died showed deterioration of the heart, kidneys, or liver. This disintegration of the solid organs was probably responsible for the increase in the amounts of uric acid and creatinine with the onset of polyneuritis.

Table I. *Results are given in milligrams per 100 cc. of blood.*

Hen number	Days on diet	Non-protein nitrogen	Creatinine	Sugar	Uric acid	Weight lbs. oz.	Remarks
1	0	45.29	1.09	176.56	5.10	5 7	After 87 days paralysis developed. Recovered on yeast-rice diet. In 121 days xerophthalmia ¹ developed. Died 9 days later.
	24	42.38	1.38	269.36	8.65	5 8	
	40	39.58	1.33	174.84	4.47	5 5	
	55	40.93	1.20	228.83	5.31	5 4	
	69	33.38	1.41	217.08	5.53	4 4	
2	0	35.68	1.03	237.68	5.89	—	Paralysis developed in 86 days. Put on yeast-rice diet and recovered.
	10	37.53	1.19	190.67	4.96	7	
	44	51.81	1.08	263.99	5.68	6 12	
	60	41.44	1.22	198.90	7.05	6 5	
3	0	41.80	2.02	176.98	4.75	6	Paralysis appeared in 74 days. Recovered on rice-yeast diet.
	10	37.20	1.38	178.06	3.98	6	
	33	44.58	1.35	180.60	1.99	5 12	
	44	42.60	0.97	254.48	7.61	5 1	
4	0	36.34	1.45	229.30	2.90	7	Had paralysis in 80 days. Died. Autopsy revealed degenerated liver.
	33	40.70	1.35	198.25	2.05	6 5	
	44	25.94	1.03	229.57	7.23	6 6	
5	0	42.48	1.46	214.70	2.24	5 12	Last sample taken 2 days after paralysis occurred. Died. No organic disturbance shown on autopsy.
	moulting 25	40.22	1.66	221.84	13.01	3 14	
6	4	36.43	1.62	248.62	6.50	6 7	Paralysis appeared in 28 days. Died in 37 days. Heart and kidneys degenerated.
	moulting 22	45.99	2.25	241.43	13.98	5 4	
7	0	37.51	1.51	239.34	8.56	6 12	Developed paralysis in 45 days. Recovered on yeast-rice diet.
	moulting 42	32.36	1.36	238.65	9.50	4 11	
8	0	34.44	1.60	218.19	1.98	5 3	Two days after last sample was taken, hen died. Autopsy showed an oedema between leg and body and degenerated liver.
	moulting 22	23.73	2.48	198.64	2.29	5	
	28	59.51	3.10	268.63	7.51	4 9	

¹ [McCollum and Simmonds, 1917.]

When the hens became so paralysed as to be unable to walk about they were fed 30 g. yeast per day and as much boiled rice as they would eat. They showed marked improvement at once although they did not gain much in weight after the first day. The hens which ate the yeast rice mixture voluntarily improved more rapidly than those which were forcibly fed. All of them were able to stand and walk about after a few days. About a week later while still on the rice yeast diet they began to lay again, and continued laying an egg every week or ten days until the end of the experiment.

SUMMARY.

1. A method for drawing blood is given by which the chickens are not injured.
2. The tendency of the blood to clot varies widely in different chickens and increases with the number of punctures during a single blood-letting.
3. There is a marked increase in the uric acid and creatinine content of the blood with the onset of polyneuritis.
4. Autopsy showed degeneration of the solid organs of the chickens upon which the polyneuritis was allowed to run its course.
5. Chickens which were fed on yeast after they developed polyneuritis showed marked increase in vitality, although there was very little increase in weight.
6. The variation of the two groups of chickens with reference to the development of polyneuritis shows that such experiments should be so regulated that the moulting season is avoided. (See appended table.)

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XLII. THE PURIFICATION OF INSULIN AND SOME OF ITS PROPERTIES.

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for Medical Research.*

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THE discovery of Banting and Best [1922] of the pancreatic hormone, insulin, which affects the metabolism of the normal animal so profoundly, and which has proved to be of such great practical importance in the treatment of human diabetes, has aroused widespread interest.

The present communication describes attempts to obtain the active principle in as pure a condition as possible, and experiments which have been made with the hope of determining some of its general chemical characteristics.

The insulin employed in this research has been made from fresh ox pancreas by a method which is essentially that of Collip [1923] with certain slight modifications which we have found advantageous, and is described in detail in the experimental section of this paper. The active fraction, which we may call "crude insulin," is obtained in the form of a white, hygroscopic powder, very readily soluble in water, in which it dissolves to form a pale straw-coloured solution which is slightly acid to litmus. The yield varies considerably from preparation to preparation, but the average yield from 21 batches, in which a total weight of 103.6 kilos. pancreas was worked up, was 0.718 g. per kilo. The estimation of the activity of these preparations has been based on the original "Toronto rabbit-unit," *i.e.* the dose necessary, when injected subcutaneously, to lower the blood sugar of a 2 kilo. rabbit, which has fasted for 16–24 hours, from a normal value of about 0.1 % to about 0.04 % and to cause typical hypoglycaemic convulsions within four hours.

Our own experience confirms the observations of McCormick, Macleod, Noble and O'Brien [1923] that the rabbit test is by no means quantitative. We find that rabbits, even after the same preliminary preparation by starving, vary greatly in sensitiveness to similar doses of insulin. The same rabbit will usually, though certainly not always, suffer about the same fall in blood sugar whenever it is given identical doses of insulin.

It is, therefore, possible, by using large numbers of animals, and particularly animals whose reactions are well-known from repeated experiments, to form a fairly accurate opinion as to the relative activities of various insulin preparations, though a really quantitative method of estimation is not yet available.

The dose of "crude insulin" necessary to cause hypoglycaemic convulsions in a 2 kilo. rabbit has been, in our most active preparations, 5 mg. or less, but more usually it is about 10 mg., and we get, on an average, a "rabbit unit" from about 15 g. pancreas.

Over 50 % of "crude insulin" consists of inorganic salts, and it gives positive reactions for chlorides and phosphates.

The following reactions are also positive: biuret, Millon (tyrosine), glyoxylic acid (tryptophan), Pauly (iminazole ring), Molisch (carbohydrate) and Seliwanoff (fructose). A solution in caustic soda, to which lead acetate has been added, blackens on heating, indicating the presence of sulphur.

It is obviously a very complex mixture, and it is impossible to associate any of its reactions with the physiologically active material.

The hormone is precipitated from an aqueous solution of "crude insulin" by a variety of reagents, such as uranium acetate and phosphotungstic acid, but the most practically useful precipitant is picric acid, which removes it from dilute aqueous solution quantitatively. The picrate, which is an amorphous, lemon-yellow powder, weighs from one-twelfth to one-fourteenth of the amount of crude insulin taken. It is only very slightly soluble in water but can be dissolved in dilute (*N*/15) di-sodium hydrogen phosphate solution for testing on rabbits. A rabbit unit of the picrate is about 1 mg.

It has been found to be quite easy to convert this insoluble picrate into a soluble hydrochloride. It is ground up with a little absolute alcohol until a uniformly fine suspension is obtained. A small quantity of an alcoholic solution of hydrochloric acid is then added and the mixture is stirred for a few minutes and dry ether is finally added. The hydrochloride separates out as an almost white powder which may be washed free from picric acid with dry ether. It is a white, perfectly non-hygroscopic and stable powder, readily soluble in water, and is obtained in a yield of 75-80 % of the picrate. The rabbit unit of this material falls generally between 0.5 and 1 mg.

The yield of hydrochloride is therefore 1 g. from 20-25 kilos. pancreas and approximately 6 % of the "crude insulin." Within the limits of determination possible by the admittedly inaccurate rabbit test, we have not been able to detect any loss of activity in preparing the hydrochloride from "crude insulin."

"Crude insulin" contains some material which causes a moderate, though transient, fall in the blood pressure of an anaesthetised animal. This substance is completely eliminated in the purification effected by means of picric acid, and the hydrochloride consequently displays no such effect.

The chemical reactions of the hydrochloride are interesting; in contrast to "crude insulin" its solution no longer gives an immediate positive test for phosphate. Further, after oxidising the solid with a mixture of nitric and sulphuric acids the test for phosphoric acid is negative, and indicates the absence of organically combined phosphorus.

This confirms the observation of Doisy, Somogyi and Shaffer [1923] made on "insulin" purified by a different procedure. Moreover, it no longer gives

Seliwanoff's reaction for fructose. This therefore disposes of the suggestion of Winter and Smith [1923] that these reactions may be associated with the active principle.

It no longer gives any reaction for tryptophan, and the Millon reaction (tyrosine) is now very faint and atypical. It still gives a positive Molisch (carbohydrate) reaction, but it is well-known that this reaction is almost always present in protein-containing material. The biuret reaction is given strongly, the Pauly reaction is intense, and the organic sulphur reaction is quite marked. It is, in fact, a substance, exhibiting the characteristics of a protein derivative, in which, of the common tests applied in such cases, the biuret and those for the iminazole ring (histidine) and for sulphur are the only striking reactions. It should be noted that Best and Macleod [1923] state that insulin prepared from the ox and pig pancreas gives a biuret reaction, an orange colour by the xanthoproteic test and a faint purple with glyoxylic acid. They fail, however, to produce any of these reactions with insulin prepared from skate pancreas by alcoholic extraction and heat. Doisy, Somogyi and Shaffer attribute a distinct biuret reaction, faint glyoxylic acid and doubtful Millon reactions to their purest preparation.

None of these investigators appears to have tried the Pauly and sulphur tests which are the characteristic reactions of the preparation from ox pancreas; the others, with the exception of the biuret, vanish or become insignificant when insulin is purified by precipitation with picric acid. It may be mentioned, in passing, that small amounts of insulin were prepared in the course of this investigation from the pancreas of the dog-fish and of the skate by the method described in this paper, and in both cases the "crude insulin" gave a very definite biuret reaction.

Whether the sulphur is present as cystine, or as some other organic sulphur-containing compound, is at present uncertain. After hydrolysis with hydrochloric acid under suitable conditions a solution is obtained which, after reduction, gives the sodium nitroprusside reaction, carried out according to Hopkins' instructions [1921], with considerable intensity. Hydrolysis with stronger acid, however, apparently destroys the substance which gives this reaction. Further experiments are being performed in this connection. It is impossible to obtain a positive nitroprusside reaction in a reduced solution of insulin hydrochloride itself. The sulphur in the intact substance is therefore not reducible, as it is, for example, in glutathione.

Doisy, Somogyi and Shaffer [1923] have shown that insulin may be precipitated from aqueous solution at what is called its "isoelectric point" by adjusting the p_{II} of the solution to about 5. If a solution of the hydrochloride be treated in this manner a precipitate is formed which has a rabbit unit value of about 0.3 mg.

The whole of the active substance, however, is not precipitated, at any rate from a 2 % solution of the hydrochloride. This precipitate, like the hydrochloride, gives strong Pauly and sulphur reactions.

The hydrochloride dissolves in water giving a solution with a p_H of about 4, and the "isoelectric point" precipitate is produced by adding alkali to such a solution. A precipitate is also formed by adding strong acid to the hydrochloride solution, until it contains about 3.3 % HCl. A flocculent white precipitate is formed which is somewhat more active than the "isoelectric point" precipitate. 0.25 mg. of this substance proved to be a rabbit unit, and it is the most potent preparation so far obtained.

In this case also the precipitation of the active principle is not complete. The precipitate, again like the hydrochloride, gives strong Pauly and sulphur reactions.

Experiments have been made to ascertain the stability of insulin to acid and alkali. It proves to be rather surprisingly stable to acid. If an aqueous solution of "crude insulin" is heated to 100° for 10 minutes in a boiling water-bath a great deal of its activity is destroyed. But in $N/10$ H_2SO_4 it will stand this treatment for 30 minutes without detectable loss of activity. After one hour of such treatment it is obvious that some destruction has taken place, and still more after two hours, but even then a considerable amount of the active principle remains.

Very different is its behaviour to alkali. Heating to 37° for 1½ hours in $N/10$ NaOH destroys its activity completely, and the same destruction is effected by heating to 100° in a boiling water-bath for 10 minutes in $N/10$ Na_2CO_3 . It is more slowly inactivated by digestion in $N/10$ Na_2CO_3 at 37°, retaining a considerable amount of activity after two hours under these conditions.

It is noteworthy that not only is "insulin" readily destroyed by trypsin, thus confirming the original supposition of the Toronto workers, on which they based their methods for its isolation, but it is also decomposed with remarkable ease and rapidity by pepsin.

An attempt to filter a solution of "crude insulin" through a collodion sack under pressure showed that only minute amounts of the active principle passed through.

As before mentioned, addition of uranium acetate to a solution of crude insulin precipitates the active principle completely. A point of some practical importance was established when it was found that the insulin could be washed off the precipitate by means of weakly alkaline solutions of disodium hydrogen phosphate. It is evidently adsorbed on the precipitate of uranyl phosphate in virtue of its colloidal nature. For human use insulin has to be sterilised by filtration through Berkefeld filters, and serious losses of activity had been experienced when solutions of "crude insulin" were so filtered. These solutions are faintly acid, with a p_H not far removed from the "isoelectric point." In consequence of the uranium acetate experiment it was found that if the p_H of a solution of insulin is first adjusted to approximately 7.5 it may be filtered through a candle without detectable loss. It is obvious that filtration of acid solutions is bound to be a risky procedure in view of

the formation of precipitates, not only at the "isoelectric point," but also in stronger acid.

A review of the results obtained enables us to draw certain general conclusions.

The *complete* precipitation of insulin from dilute solution by picric acid, its ready adsorption, its failure to pass through an ultra-filter, its rapid digestion by trypsin, and particularly by pepsin, all indicate that it is most probably a substance having a very complex structure and protein-like in its nature. The hope of its isolation as a chemically pure substance becomes slender, and of its synthesis very remote, by methods at present at our disposal.

It is certainly much more complex than the active principles of the pituitary posterior lobe, probably even more so than "secretin." It is more analogous in complexity to the toxins, to ricin, or to Vaughan's toxic protein derivatives.

It is obvious that the administration of insulin to patients by the mouth is impossible on account of its rapid destruction by the digestive enzymes, and the relatively large size of its molecule makes it unlikely that absorption from mucous surfaces, such as that of the nasal passages, a method which has recently proved useful in controlling diabetes *insipidus* with pituitrin, will be practically useful in the case of insulin.

The fact that great physiological activity is associated with both the "isoelectric point" precipitate and the strong hydrochloric acid precipitate, described in this paper, suggests that the active principle is present in such a preparation as the "insulin hydrochloride" in quite indeterminate amount, associated with other substances of approximately similar physical properties. In this case it is impossible to decide whether the characteristic Pauly and sulphur reactions bear any relation to the actual active principle. It is, however, intriguingly interesting that the Pauly reaction should be so strongly positive. It occurs so frequently in connection with preparations of highly physiologically potent substances, such as those of the oxytocic and pressor principles of the pituitary gland, that one is tempted to suspect that derivatives of histidine are apt to be endowed with various intense physiological activities; but, at present, this is pure speculation.

EXPERIMENTAL.

Preparation of "Crude Insulin."

Five kilos. of perfectly fresh ox pancreas are minced through a sausage machine into 5 litres of 95 % alcohol, previously cooled to -3° . Not more than two hours should elapse between the slaughtering of the animals and the mincing of the tissue into alcohol. It is advantageous to strain off the minced tissue on a fine sieve, squeeze out as much of the alcohol as possible by hand, remince the tissue into the alcohol and repeat the mincing a third time in order to get as good

disintegration as possible. The mixture is then allowed to stand for two hours at room temperature with frequent stirring. It is then filtered through large folded filters of thick "Chardin" paper. As soon as the main bulk of the liquid, which may be slightly turbid, is filtered, the thick paste on the paper is transferred to a suitable press and as much liquid as possible is squeezed out. The press liquor and filtrate are mixed. This filtration is tedious, but is completed in three to four hours. Approximately 5.5 litres of filtrate are collected. $1\frac{1}{2}$ volumes (i.e. 8.25 litres) of 95 % alcohol are now added. A white precipitate appears and the liquid is placed in a cold room at -3° overnight.

The liquid is then filtered through "Chardin" folded filters in the cold room. This filtration proceeds without difficulty, giving a perfectly clear, yellow solution. The filtrate is transferred in quantities of $2\frac{1}{2}$ -3 litres to a battery of five 5-litre round-bottomed flasks, and the alcohol is distilled off *in vacuo*, the temperature of the water-baths being kept at 45° .

When most of the alcohol has been removed the liquid becomes turbid owing to the separation of fat. The residual liquid in the five flasks is collected in one flask and further distilled *in vacuo* with the water-bath at 45° . This removes the remainder of the alcohol, and the fat usually separates in buttery lumps. The distillation is continued until the volume of the aqueous residue is about 250 cc. It is then filtered from fat on a Buchner funnel and the main bulk of the fat is shaken out of the flask and squeezed as free from solution as possible. (Occasionally the fat does not solidify but remains floating in the concentrated aqueous solution as a thick, yellow oil. When this happens, the entire contents of the flask are transferred to a separating funnel and shaken with a small quantity of light petroleum. This dissolves the fat readily, and the aqueous layer can be tapped off without loss.) The aqueous solution is then made up to 80 % with absolute alcohol; that is to say, to every 100 cc. of solution are added 400 cc. of absolute alcohol. A yellow syrup separates in fine drops and falls to the bottom of the solution which is then placed in the cold room overnight.

After standing, the syrup and a certain amount of solid matter adhere to the bottom of the containing vessel, preferably a tall, narrow cylinder, and the supernatant liquid is decanted without difficulty and made up to 93 % by the addition of two volumes of absolute alcohol. A somewhat scanty white precipitate forms and the liquid is placed in the cold room for 15-20 hours. The precipitate has now settled to the bottom as a white granular powder, the supernatant liquid being almost, if not quite, clear. The latter is removed by means of a siphon except for a small amount left covering the precipitate, which is then stirred up with the residual alcohol and poured into centrifuge tubes. The precipitate is centrifuged down, washed once or twice with absolute alcohol and once or twice with dry ether in the centrifuge. After pouring off the ether the tubes containing the precipitate are quickly transferred to a vacuum desiccator and dried over sulphuric acid. A yield of from 3-4 g. is obtained in the form of a white, hygroscopic powder.

Preparation of the Active Picrate and Hydrochloride.

A 1.5 % aqueous solution of "crude insulin" is made. It is freed from any small amount of suspended matter by centrifuging—filtration, even through paper, is apt to be attended by loss of activity—and then half its volume of a saturated aqueous solution of picric acid is added. An immediate flocculent, amorphous precipitate is formed which settles down rapidly to the bottom of the liquid, leaving a fine suspension in the supernatant fluid. After standing for one or two days at ordinary room temperature the supernatant liquid becomes quite clear and is poured off. A little water is then added to the precipitate which is now adhering to the bottom of the beaker. The picrate is rubbed up with a glass rod and the suspension is then transferred to a centrifuge tube and spun down. It is washed with repeated additions of water in the centrifuge until finally the supernatant liquid displays a slight turbidity on the addition of a few drops of saturated aqueous picric acid. This indicates that the picrate is beginning to go into solution. It is then suspended in a small volume of water and filtered on a small Buchner funnel and finally placed on porous pot and dried in a vacuum desiccator over H_2SO_4 . A lemon-yellow amorphous powder is obtained in a yield of about 7.5 % of the crude insulin taken.

The precipitation should be carried out in dilute solution as indicated, although it is permissible to use a 2 % solution of "crude insulin," adding equivalently larger amounts of picric acid solution. If precipitation from strong solutions is attempted other picrates are precipitated also which are inert physiologically.

The mother liquor from the precipitation of the picrate has been examined in numerous batches for blood-sugar lowering material. It has been evaporated *in vacuo* to a convenient bulk, then faintly acidified with hydrochloric acid, the picric acid shaken out with ether, and finally injected into a rabbit. We have never succeeded in finding a trace of insulin in this way. For instance, 1 g. of "crude insulin," which had a rabbit unit of 10 mg. was precipitated with picric acid in the manner described. Half the filtrate, representing 0.5 g. of the original powder, or 50 rabbit units, was concentrated *in vacuo* to about 10 cc. It was then acidified with 2 cc. $N \text{ H}_2\text{SO}_4$ and the picric acid was removed by shaking out with ether. The ether was removed from the solution *in vacuo* and then, after adding 2 cc. $N \text{ Na}_2\text{CO}_3$, the whole solution was injected into a 2.4 kilo. rabbit subcutaneously. The blood sugar values, taken every hour, were as follows:

Blood sugar, normal	0.096 %
" " 1 hour after injection	0.093
" " 2 hours " "	0.101
" " 3 " " "	0.105

It will be seen that there is no fall in the blood sugar value, indicating that the removal of insulin by means of picric acid is quantitative.

The proper washing of the picrate is of great importance. It is more practicable to do the washing in a centrifuge tube since the picrate tends to clog on a filter paper. If any mother liquor remains in the wet precipitate it will be found that, on drying, the picrate, instead of remaining pale yellow and easily powderable, forms a hard, dark brown mass, rather like shellac. In this condition it is quite unsuitable for conversion into the hydrochloride. If a batch of picrate is found in this state after drying it should be dissolved in $N/10$ Na_2CO_3 and the clear solution should then be acidified with HCl until just acid to litmus. After the addition of a small amount of picric acid solution the reprecipitated picrate is washed thoroughly and recovered as described above. It may be noted that on reprecipitation in this manner the picrate is in a somewhat more easily filterable condition and may be collected and washed on a filter paper.

If a sufficient quantity of picrate is available this reprecipitation is to be recommended as giving a slightly purer product, which is also in better physical condition for the subsequent transformation into hydrochloride.

This is accomplished by grinding up 1 g. of picrate with 6 cc. absolute alcohol in a mortar until a very fine uniform suspension is obtained. It is then transferred to a centrifuge tube of about 40 cc. capacity and washed in with 2 cc. absolute alcohol. 4.5 cc. of a solution of dry HCl (5 % by weight) in absolute alcohol are added to the suspension which is stirred for about five minutes. About 30 cc. of dry ether are then added and the hydrochloride settles rapidly as a white amorphous powder. After centrifuging, the clear liquid is poured off and the precipitate is washed twice with dry ether. It is then transferred in the tube to a vacuum desiccator containing sulphuric acid and the residual ether is removed by cautious evacuation. The hydrochloride, an almost white, non-hygroscopic powder, is obtained in a yield of about 75-80 % of the picrate.

In a typical preparation 4.64 g. of "crude insulin" were taken. A rabbit unit of this material was 15 mg. 0.3281 g. picrate was obtained, of which 1 mg. was a rabbit unit. This yielded 0.2461 g. of hydrochloride which on first testing caused convulsions in a rabbit in a dose of 0.5 mg. On retesting, this dose just failed to produce convulsions although the blood sugar approached the convulsant level, while a rabbit receiving 1 mg. had severe convulsions. The total number of rabbit units in the "crude insulin" was 309. The assay of the picrate indicated 328 "units," while if 0.75 mg. be taken as the "rabbit unit" of the hydrochloride the yield from the picrate was quantitative.

Elimination of Depressor Constituent of "Crude Insulin" by means of the Picrate Purification.

In this experiment a portion of a batch of "crude insulin" which had a rabbit unit of 10 mg., was converted into the hydrochloride of which 0.75-1.0 mg. proved to be a rabbit unit.

A cat, with vagi intact, under ether anaesthesia, was used to determine the effect of the "crude insulin" and the hydrochloride on the blood pressure.

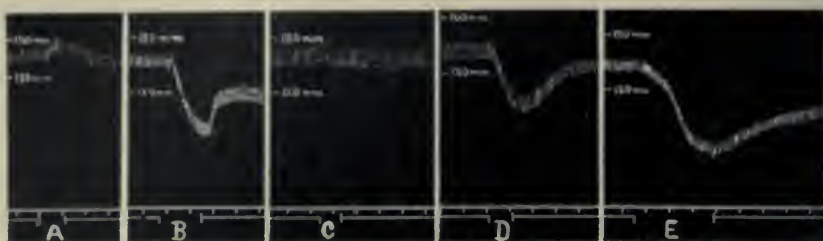


Fig. 1.

The following injections into the femoral vein were given

- | | | | | |
|----|--------|-------|-------|--------------------|
| A. | At 4.0 | p.m.: | 2 mg. | hydrochloride. |
| B. | " 4.2 | " | 20 " | " "crude insulin." |
| C. | " 4.6 | " | 5 " | hydrochloride. |
| D. | " 4.8 | " | 20 " | " "crude insulin." |
| E. | " 4.13 | " | 50 " | " "crude insulin." |

At A, B and D, therefore, two rabbit units of insulin were given and at C and E five rabbit units.

The hydrochloride, then, under these conditions, had no effect on the blood pressure, while corresponding doses of "crude insulin" produced marked falls in blood pressure.

The "Isoelectric Point" Precipitate.

0.1045 g. of "insulin hydrochloride" was dissolved in 4 cc. of water in a centrifuge tube. 0.4 cc. $N \text{ Na}_2\text{HPO}_4$ was added, drop by drop. It was found that after this amount had been added no further precipitate was formed. The p_{H} of the solution was 5.7. The precipitate was formed very promptly; standing in the cold room overnight produced no more. It was centrifuged off, washed with absolute alcohol and finally with dry ether. It was then dried by placing it in a vacuum desiccator over H_2SO_4 and evacuating cautiously. The dry precipitate, a white powder, weighed 0.0353 g.

It was dissolved in $N/15 \text{ Na}_2\text{HPO}_4$ solution for testing on rabbits. The rabbit unit of the hydrochloride was 0.5 mg. and that of the precipitate 0.33 mg., so that 50 % of the activity had been removed from the solution and the precipitate was about $1\frac{1}{2}$ times as active as the hydrochloride from which it had been prepared.

This ratio was checked by testing the residual activity of the solution from which the precipitate was obtained.

*The Precipitate formed by the Addition of strong Hydrochloride Acid
to a Solution of "Insulin Hydrochloride."*

0.13 g. of hydrochloride was dissolved in 10 cc. water. 10 % hydrochloric acid was added gradually until no further precipitate was formed. This required 5 cc. of the acid. After standing in the cold room overnight the precipitate was centrifuged down, washed with a little 3.3 % HCl, and dried in a vacuum desiccator. The weight of the precipitate was 0.02 g.

It was dissolved in water for the rabbit test. It was not quite completely soluble in water, a small amount of flocculent material remaining suspended in the liquid.

The mother liquor was nearly neutralised with NaOH and then treated with aqueous picric acid solution and the residual material recovered as picrate. It weighed 0.0815 g.

The rabbit unit of the hydrochloride was 0.5 mg., and that of the precipitate 0.25 mg. The precipitate was therefore twice as active as the hydrochloride from which it had been prepared, and 32 % of the total activity had been precipitated.

Stability of Insulin to Acid.

A batch of "crude insulin" of which the rabbit unit was 10 mg. was used in these experiments.

A solution of the "crude insulin" containing 10 mg. per cc. was made in $N/10$ H_2SO_4 and divided into 1 cc. portions. These were heated in test-tubes in a boiling water-bath for various periods, and then cooled, neutralised with $N/10$ Na_2CO_3 , and injected subcutaneously into rabbits. The following results were obtained:

Ten minutes' heating. Rabbit weighed 1.6 kilo.:

Blood sugar, normal	0.107 %
" " 1 hour after injection	0.047
" " 2 hours " "	0.023

The animal had convulsions in 2 hours and was recovered by injecting glucose.

Thirty minutes' heating. Rabbit weighed 2.35 kilos.:

Blood sugar, normal	0.111 %
" " 1 hour after injection	0.036
" " 2 hours " "	0.029

Animal had convulsions in 2 hours and was recovered by injecting glucose.

One hour's heating. Rabbit weighed 1.1 kilo.:

Blood sugar, normal	0.090 %
" " 1 hour after injection	0.061
" " 2 hours " "	0.056
" " 3 " " "	0.065

Two hours' heating. Rabbit weighed 1.1 kilo.:

Blood sugar, normal	0.088 %
" " 1 hour after injection	0.055
" " 2 hours " "	0.072
" " 3 " " "	0.093

The last two results show that slow destruction of the active principle is occurring.

An aqueous solution of the same batch of "crude insulin" containing 10 mg. per cc. was heated to 100° for ten minutes. 1 cc. of the solution was then injected subcutaneously into a rabbit weighing 1.75 kilo.:

Blood sugar, normal	0.108 %
" " 1 hour after injection	0.092
" " 2 hours " "	0.069
" " 3 " " "	0.081
" " 4 " " "	0.090

Evidently in such a faintly acid solution—the p_H is about 6.1—insulin is quite unstable at 100°.

Stability to Alkali.

The same batch of insulin was used as in the experiments on stability to acid.

10 mg. were dissolved in 1 cc. $N/10$ NaOH and heated to 37° for 1½ hours. The solution was then neutralised with 1 cc. $N/10$ HCl and injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.101 %
" " 1 hour after injection	0.105
" " 2 hours " "	0.101

No trace of activity is left.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated to 37° for one hour. After neutralisation with 1 cc. $N/10$ HCl the solution was injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.114 %
" " 1 hour after injection	0.066
" " 2 hours " "	0.048
" " 3 " " "	0.035

The animal had convulsions in three hours and was recovered by the injection of glucose.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated in a boiling water-bath to 100° for ten minutes. After neutralisation the solution was injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.113 %
" " 1 hour after injection	0.107
" " 2 hours " "	0.113
" " 3 " " "	0.129

This result indicates complete destruction of the active principle.

Action of Trypsin.

0.5 g. of a commercial sample of trypsin was dissolved in 10 cc. $N/10$ Na_2CO_3 and 20 mg. of the same "crude insulin" which was used in the previous experiments on the stability to acid and alkali was dissolved in 2 cc. water.

The insulin solution was divided into two equal portions of 1 cc. each and to one of them was added 1 cc. of the trypsin solution, and to the other 1 cc. of a portion of the trypsin solution which had previously been heated to 100° for 30 minutes. A drop of chloroform was added to each solution.

After incubation at 37° for two hours they were injected into two rabbits.

(1) Trypsin experiment. Rabbit weighed 3.5 kilos.:

Blood sugar, normal	0.092 %
" " 1 hour after injection	0.104
" " 2 hours " "	0.101
" " 3 " " "	0.104

(2) Heated trypsin control. Rabbit weighed 3.25 kilos.:

Blood sugar, normal	0.101 %
" " 1 hour after injection	0.059
" " 2 hours " "	0.066
" " 3 " " "	0.074

Trypsin, therefore, promptly destroys insulin.

Action of Pepsin.

1st experiment. 0.3 g. of a commercial pepsin preparation was dissolved in 50 cc. N/500 HCl. A portion of this solution was heated to 100° for 30 minutes.

20 mg. "insulin hydrochloride," of which the rabbit unit was 0.8 mg., were dissolved in 5 cc. water.

(1) To 2.5 cc. of the insulin hydrochloride solution were added 2.5 cc. of the pepsin solution.

(2) To 2.5 cc. of the insulin hydrochloride solution were added 2.5 cc. of the heated pepsin solution.

To each of the solutions were added a few drops of chloroform. They were then placed in the hot room at 37° for 16 hours.

1 cc. of (1) (i.e. 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2.4 kilos.:

Blood sugar, normal	0.123 %
" " 1 hour after injection	0.129
" " 2 hours " "	0.124

1 cc. of (2) (i.e. 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2.3 kilos.:

Blood sugar, normal	0.100 %
" " 1 hour after injection	0.077
" " 2 hours " "	0.041

The animal had convulsions 2½ hours after the injection and was recovered by injecting glucose.

The experiment shows a complete destruction of insulin by pepsin.

2nd experiment. It was desired, if possible, to demonstrate the action of pepsin in a shorter time than in the previous experiment.

A 1 % solution of commercial pepsin of a different source from that employed in the first experiment was made in N/10 HCl. A portion of this solution was heated to 100° for 30 minutes. A solution of the same specimen of "insulin hydrochloride" containing 4 mg. per cc. was prepared.

(1) To 3 cc. of the insulin hydrochloride were added 3 cc. of the pepsin solution.

(2) To 2 cc. of the insulin hydrochloride were added 2 cc. of the heated pepsin solution.

A few drops of chloroform were added to the solutions, which were then placed in the hot room at 37°.

After one hour 1 cc. of solution (1) was removed, neutralised with Na_2CO_3 and injected into a rabbit weighing 2.2 kilos. After two hours a similar sample was withdrawn and injected into a rabbit. As the destruction of the insulin was found to be complete at the end of the first hour only the blood sugar record of the first rabbit is given:

Blood sugar, normal	0.107 %
" " 1 hour after injection	0.113
" " 2 hours " "	0.114
" " 3 " " "	0.126

After four hours a 1 cc. sample was withdrawn from the control experiment (2) and, after neutralisation, injected into a rabbit weighing 1.9 kilo.:

Blood sugar, normal	0.120 %
" " 1 hour after injection	0.074
" " 2 hours " "	0.063

The animal was then given an injection of glucose and the experiment discontinued.

The insulin was completely destroyed by pepsin in one hour.

Berkefeld Filtration of "Crude Insulin."

1.04 g. of crude insulin was dissolved in 100 cc. water. The p_{H} of this solution was 6.1.

It was then sucked through a kieselguhr candle, except for a small quantity (6.5 cc.) which was held back for tests. Owing to traces of alkali in the candle the p_{H} of the filtered solution had risen to 6.6. The filter was washed by drawing 50 cc. $N/10 \text{ Na}_2\text{HPO}_4$ through it.

1 cc. of the unfiltered solution was injected into a rabbit weighing 1.6 kilo.:

Blood sugar, normal	0.111 %
" " 1 hour after injection	0.046
" " 2 hours " "	0.030

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose after the 2nd hour blood sample had been taken.

1 cc. of the filtered solution was injected into a rabbit weighing 2.3 kilos:

Blood sugar, normal	0.115 %
" " 1 hour after injection	0.085
" " 2 hours " "	0.071
" " 3 " " "	0.087
" " 4 " " "	0.102

0.5 cc. of the sodium phosphate solution was injected into a rabbit weighing 2.5 kilos:

Blood sugar, normal	0.106 %
" " 1 hour after injection	0.041
" " 2 hours " "	0.031
" " 3 " " "	0.032

The animal recovered from convulsions without requiring an injection of glucose.

It is apparent that nearly all of the active material was adsorbed by the kieselguhr filter, and that the adsorbed substance was recovered by passing the alkaline solution of sodium phosphate through the filter.

Berkefeld Filtration of "Crude Insulin" in Alkaline Solution.

0.547 g. of the preparation used in the previous experiment was dissolved in 54.7 cc. $N/10 Na_2HPO_4$. The p_H of this solution was 7.2.

A portion was reserved unfiltered for control tests and the rest was passed through a kieselguhr filter.

The p_H of the filtered liquid was 7.4 owing to traces of alkali in the candle. The filtrate was found to be sterile by bacteriological tests.

1 cc. of the unfiltered solution was injected into a 2.5 kilo. rabbit.

Blood sugar, normal	0.090 %
" " 1 hour after injection	0.040
" " 2 hours " "	0.047

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose.

1 cc. of the filtered solution was injected into a rabbit weighing 2.1 kilos.:

Blood sugar, normal	0.116 %
" " 1 hour after injection	0.052
" " 2 hours " "	0.046

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose.

All the rabbits used throughout these experiments were starved for 16-24 hours before the tests were made.

The blood sugars were estimated by the method of Shaffer and Hartmann [1920].

I am much indebted to Dr H. H. Dale for constant interest and helpful criticism throughout the course of the work and also for his kindness in performing the blood pressure experiment.

I wish to express my sincere thanks to Mr H. P. Marks for having carried out a large number of blood sugar determinations and to Mr W. W. Starling for valuable help in the preparation of "crude insulin."

SUMMARY.

1. A method of purifying crude preparations of insulin is described. It consists in precipitating the active material with picric acid and converting the insoluble picrate into a soluble hydrochloride by means of an alcoholic solution of hydrochloric acid.

2. This hydrochloride is an almost white powder of which 0.5-1 mg. will lower the blood sugar of a 2 kilo. rabbit, which has been starved for 24 hours, to about 0.04 % and cause typical hypoglycaemic convulsions.

3. The characteristic reactions given by this substance are the biuret, Pauly and organic sulphur tests. The glyoxylic acid reaction is negative, and the Millon test is very faint and atypical. It contains no phosphorus.

4. From its solutions two precipitates may be obtained; one by the addition of acid and the other by the addition of alkali. The first is about twice and the second about one-and-a-half times as active as the original hydrochloride.

5. It is shown that both pepsin and trypsin destroy insulin.

6. Insulin is relatively stable to acid, but is decomposed with ease by alkali.

7. Insulin, in faintly acid solutions, is very readily adsorbed, but it may be filtered through Berkefeld filters without appreciable loss if the solution is first made weakly alkaline.

8. Insulin appears to be a very complex protein derivative.

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XLIII. THE ESTIMATION OF AMMONIA AND UREA IN URINE AND OTHER FLUIDS.

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(Received March 15th, 1923.)

F. W. FOREMAN [1920] has used steam alcoholic distillation to separate volatile bases from amino acids, estimations being afterwards made by his well-known method of titrating in alcohol. He has since applied the method to mixtures of urea and ammonia, and finds that it is applicable to urine¹.

The fact that when a mixture of urea and ammonia is steam distilled in alcohol only the ammonia distils over during a certain stage of the distillation has been made use of in the Cambridge Biochemical Laboratory—at Mr Foreman's suggestion—to estimate ammonia and urea in solutions containing either or both. The method is so very rapid and easy to handle that it should be generally employed. Its use can only be attended with confidence however if the conditions for the distillation are carefully defined. It is the purpose of this paper to show that with due attention to conditions it can be made absolutely reliable. When it is borne in mind that a single determination occupies only a few minutes, the advantages of the method over the aeration and other methods at present in use will be sufficiently obvious.

METHOD.

The apparatus consists of a steam boiler, a large (1 litre) distilling flask and a condenser. The distilling flask, which is placed at 45° to the horizontal, is fitted with a rubber cork carrying a tap funnel containing an alcoholic solution of caustic potash coloured with phenolphthalein; and a glass tube which is bent so that it dips right down into the lowest part of the flask. This tube is connected by a movable piece of rubber tubing to the steam boiler. The side tube of the flask is connected to the condenser, which is arranged as nearly vertical in position as possible. A collecting flask containing standard HCl is placed so as to receive the distillate. A sample (5 to 10 cc.) of the fluid under investigation is taken and mixed with (90 cc. of) alcohol and placed in the distilling flask. The boiler is heated but it is not connected to the distilling flask until a vigorous jet of steam is issuing from the connecting tube. The coloured alcoholic potash solution is next run in from the tap funnel until the fluid in the distilling flask is just alkaline to phenolphthalein. If necessary more potash may be added in this way during

¹ Private communication.

the distillation. This method of rendering the fluid alkaline in order that ammonia may be distilled off has been found necessary in order to ensure that no leakage of ammonia occurs. The fluid soon boils and ammonia and other volatile bases are distilled over into the collecting flask, where they neutralise the acid placed there; and are subsequently estimated by titration with CO_2 -free soda, using alizarin-red as an indicator. After a few minutes a foam or froth appears on the fluid in the distilling flask. This indicates that the alcohol has nearly all distilled off from the fluid; frothing commences when the alcohol concentration is still as high as 3 %. At the first sign of frothing, therefore, the rubber stopper of the flask is turned round so that the bent end of the steam delivery tube is turned up out of the fluid. The steam then passes through the distilling flask and condenser and carries away any ammonia present there. This "washing" is allowed to continue for $1\frac{1}{2}$ minutes and the collecting flask is then removed and titrated as described above.

Practical Notes.

1. Frothing should not be confused with the splashing and bubbling that occurs in the first few minutes. It is a steady foam that gradually spreads over the surface of the fluid. Frothing may be made very obvious by the addition of a minute amount of fat or fatty acid, but it is easily detected after a few attempts at using the apparatus.

2. The condenser tube should be arranged nearly vertically to ensure complete drainage. Otherwise the condenser must be washed through with distilled water at the end of the distillation.

3. To avoid fluid in the distilling flask being splashed over, use a large flask (about one litre) and incline it as much as possible.

Note on corrections applied to the results.

The alcohol used was 96 %; wherever alcohol content is expressed as a percentage, it is this percentage of 96 % alcohol to which we refer. For example "80 % alcohol" should read "80 cc. of 96 % alcohol in 100 cc. of fluid."

The distilled water used was not neutral to the indicator (alizarin-red) used. The correction was applied where necessary:

25 cc. distilled water found equivalent to 0.10 cc. of $N/50$ HCl.

The blank correction for the apparatus was obtained by distilling a mixture of alcohol and water until frothing:

Blank correction = + 0.2 cc. $N/50$ HCl.

The urea used was twice recrystallised. A small amount of ammonium carbonate, etc. was however still present as impurity. Thus:

4 cc. of 25 % urea yielded ammonia equivalent to 1.15 cc. $N/50$ HCl.

The correct value of the ammonium carbonate solutions was obtained by distillation in aqueous solution and by the Van Slyke modification of Folin's aeration method.

The value of ammonia is expressed throughout in terms of its equivalent standard acid.

The amount of ammonia given off before frothing depends upon the time of frothing and the rate of boiling, but these are determined by the composition and bulk of the fluid used. Our first experiments, therefore, were intended to show the relationship between percentage of alcohol, bulk of alcohol and aqueous solution, and the amount of ammonia given off before frothing.

Exp. 1. Effect of varying the percentage of alcohol with constant bulk of fluid (100 cc.) and constant volume of ammonium carbonate solution (5 cc.).

Percentage alcohol	(1)			(2)	
	Ammonia present cc. N/50 HCl	Ammonia distilled cc. N/50 HCl	Time to frothing m. s.	Ammonia distilled cc. N/50 HCl	Time to frothing m. s.
10	21.30	8.20	2 10	7.40	2 5
30	21.30	15.40	3 0	13.15	3 0
50	21.30	18.80	3 30	18.00	3 30
70	21.30	21.00	4 50	21.10	4 40
80	21.30	21.25	5 0	21.30	5 0
90	21.30	21.30	5 30	21.30	5 40
95	21.30	21.30	5 40	21.30	5 40

This experiment shows that only a proportion of 21.3 cc. of ammonia (N/50 acid) is distilled over before frothing if the percentage of alcohol is 70 or under. It is also evident that with constant total volume of fluid, increasing the percentage of alcohol increases the time up to frothing.

Exp. 2. The effect of varying the volume of aqueous solution, with constant volume of alcohol (95 cc.) and constant volume of ammonium carbonate solution (5 cc.).

Volume alcohol cc.	Volume of aqueous solution cc.	Ammonia present cc. N/50 acid	Ammonia distilled cc. N/50 acid	Calculated percentage alcohol
95	5	127.0	127.00	95
95	25	127.0	126.60	80
95	40	127.0	126.15	70
95	55	127.0	126.00	63
95	70	127.0	124.40	58
95	95	127.0	121.10	50

This experiment shows that although the large volume of 95 cc. alcohol is present, it is also necessary to have the percentage alcohol above a critical value to ensure that all the ammonia has distilled over before frothing.

Exp. 3. Effect of varying the total volume of fluid, percentage of alcohol being kept constant.

Percentage alcohol	Total volume of solution cc.	Ammonia present cc. N/10 acid	Ammonia distilled cc. N/10 acid	Time to frothing m. s.
90	100	42.60	42.65	5 20
90	50	42.60	41.50	4 0
90	100	cc. N/50 acid 42.35	cc. N/50 acid 42.15	5 20
90	50	42.35	42.30	4 0

This experiment shows that in addition to critical percentage, a critical volume is necessary to ensure that all the ammonia distils over before frothing. This critical volume varies with the amount of ammonia originally present in solution.

The first three experiments indicated that 90 % alcohol and 100 cc. total volume were values which ensured complete distillation of these quantities of ammonia before frothing; and our next step was to ascertain the range of ammonia concentration for which they held.

Exp. 4. Effect of varying the amount of ammonia, with constant percentage of alcohol (90 %) and constant total volume of fluid (100 cc.).

Ammonia present cc. N/10 acid	Ammonia distilled cc. N/10 acid	Ammonia distilled cc. N/10 acid
144.10	144.10	144.15
43.50	43.45	43.50
26.10	26.10	26.10
8.70	8.70	8.70
cc. N/50 acid	cc. N/50 acid	cc. N/50 acid
0.395	0.395	0.390

This experiment shows that with 90 % alcohol and 100 cc. total volume of fluid, any value of ammonia between 144.1 cc. (N/10 acid) and 0.395 cc. (N/50 acid) can be completely distilled over before frothing. We did not attempt to estimate still smaller quantities of ammonia, since the blank correction was greater than 50 % of this ammonia value. The highest value of ammonia estimated represents 5 cc. of saturated ammonium carbonate solution, *i.e.* the highest concentration possible. The values of 90 % alcohol and 100 cc. total fluid are therefore safe and accurate for this large range of ammonia values.

We then investigated whether the presence of urea in the solution would influence the proportion of ammonia given off before frothing.

Exp. 5. Effect of varying the percentage of alcohol, total volume of fluid (100 cc.) and the ammonia content being kept constant. The solution contains 4 cc. of 25 % urea.

Percentage alcohol	Ammonia present cc. N/10 acid	Ammonia distilled, cc. N/10 acid	
		(1)	(2)
90	144.10	144.10	144.10
80	144.10	144.05	144.05
70	144.10	143.40	143.50
60	144.10	141.60	141.65

The experiment shows that the presence of urea in solution does not apparently influence the distillation of the ammonia. With 90 % alcohol and total volume of 100 cc. the whole of the ammonia distils over before frothing (*cf.* Exp. 1).

Exp. 6. Effect of extreme ratios of urea and ammonia, with percentage of alcohol constant (90 %) and total volume of fluid constant (100 cc.).

Saturated urea solution	0.04 % urea solution	Ammonia present cc. N/50 acid	Ammonia present cc. N/10 acid	Ammonia distilled, cc.	
				(1)	(2)
5	—	0.395	—	0.395	0.400
5	—	—	144.10	144.05	144.15
—	5	0.395	—	0.390	0.395
—	5	—	144.10	144.20	144.10

This experiment shows that very small and very large amounts of ammonia can be estimated accurately in the presence of very large and very small concentrations of urea.

Time course of the Distillation.

It was necessary to ascertain for different percentages of alcohol the exact time relation of frothing to the end of ammonia distillation and the commencement of urea decomposition. This was done by finding the volume of ammonia given off at different periods after the commencement of distillation. The collecting flasks containing standard acid were changed at noted times. Volumes of ammonia (equivalents in $N/50$ acid) were plotted along the ordinates, and time along the abscissae, and the time curves thus obtained. The total volume of fluid in all cases was 100 cc.

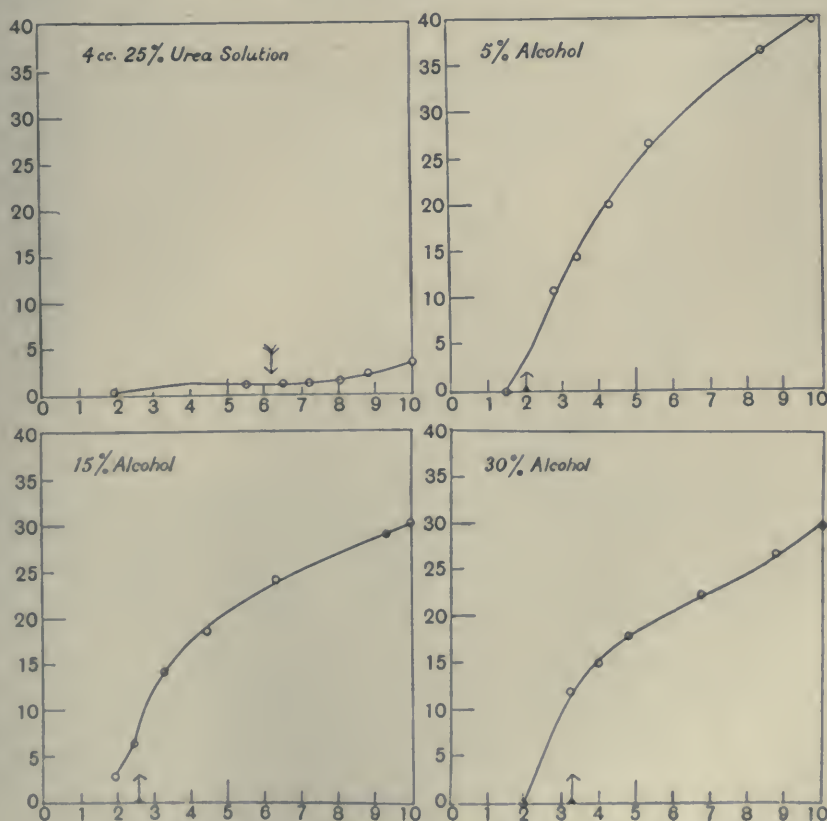


Fig. 1. Curves showing time relations of the analysis.

Abscissae = time from commencement of analysis in minutes.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Total bulk of fluid 100 cc. Ammonia content the same in all the curves except the first.

See Exp. 6.

Arrows indicate commencement of frothing.

Practical Notes.

1. The ammonia in the condenser at the time of removing a receiving flask is inevitably collected in the next flask.

2. The slight loss of ammonia that may occur on changing collecting flasks does not affect the well-marked nature of the curves.

3. The time curve for urea alone shows that the ammonia from the impurity of ammonium carbonate is distilled over completely before frothing. The value of the ammonia impurity was confirmed by Van Slyke's method, thus permitting a correction for the remaining time curves.

Exp. 7. Data for time curves. The following tables illustrate the nature of the data upon which the curves are based.

The time of frothing and the value of ammonia distilled over by that time are denoted by black figures. In each case total volume of fluid is 100 cc.; urea content is constant (4 cc. of 25 % solution) and ammonia content is constant (21.40 cc. *N*/50).

(1) Urea alone in 100 cc. 90 % alcohol		(2) Urea and ammonia in 30 % alcohol		(3) Urea and ammonia in 90 % alcohol	
Time m. s.	Ammonia distilled cc. <i>N</i> /50	Time m. s.	Ammonia distilled cc. <i>N</i> /50	Time m. s.	Ammonia distilled cc. <i>N</i> /50
2 0	0.1	2 0	0.3	2 0	5.50
4 0	0.45	3 20	12.1	4 0	18.90
6 30	1.10	4 0	15.2	5 30	21.40
7 10	1.10	4 50	18.1	6 10	21.40
8 0	1.50	6 50	22.5	6 50	21.45
8 50	2.10	8 50	26.7	7 40	21.85
10 50	5.30	11 50	33.3	9 40	24.35
13 0	10.10	15 0	40.0	11 40	27.55
5 20	15.30			15 0	32.85

N.B. 1.10 cc. represents impurity (*vide supra*).

Deductions from Time Curves.

The curves for percentages of alcohol of 70 and under (Figs. 1 and 2) show that there is no appreciable time interval between the end of ammonia distillation and the commencement of urea decomposition. The ammonia from the urea decomposition distils over before the whole of the ammonia of the ammonium carbonate has been distilled. With these low percentages it is therefore impossible to stop distillation at any definite time and obtain a correct result. This explains the discrepant results obtained through not using a sufficiently high percentage of alcohol. Frothing in these cases is of no value as a time mark. The 80 % alcohol curve shows a minimum period of zero distillation but it is not absolutely flat and must therefore be classified as unreliable for accurate work. The 90 % alcohol curve, however, shows a well-marked flat zero period, which extends over an appreciable time; and during which no ammonia distils over. Frothing (indicated by a vertical arrow) is seen to fall at the commencement of this flat zero period, and marks in this case the completion of ammonia distillation. It therefore serves as a valuable signal

for the cessation of distillation. The flat zero period represents the safety interval during which no ammonia distils over, and this is followed by urea decomposition. 90 % alcohol and a total volume of 100 cc. are therefore the conditions necessary for accurate ammonia distillations in the presence of urea.

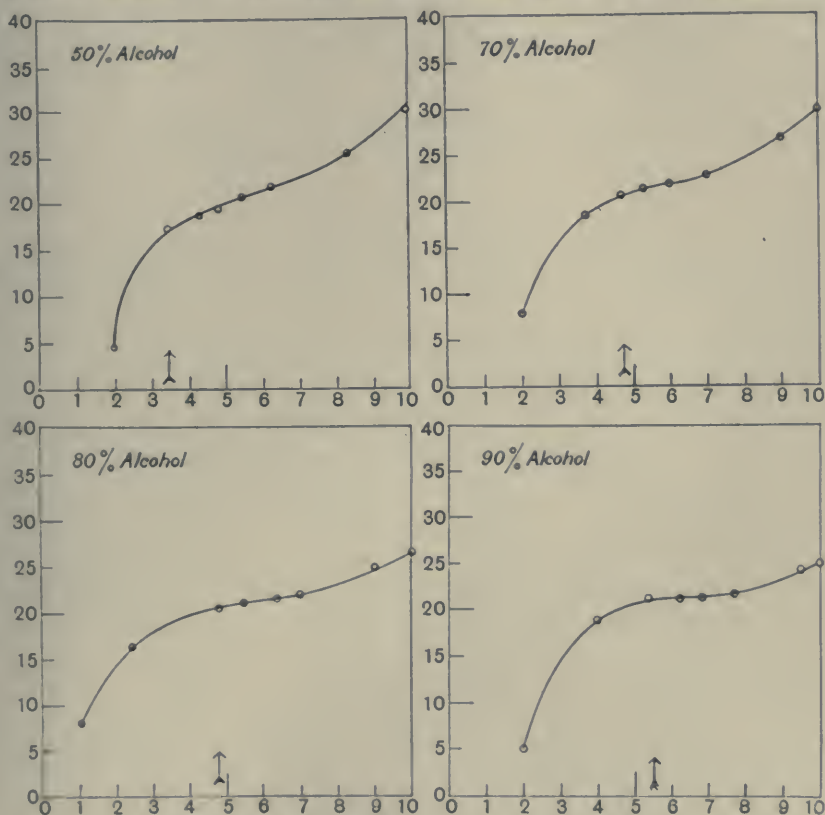


Fig. 2. Curves showing time relations of the analysis.

Abcissae — time from commencement of analysis in minutes.

Ordinates — cc. $N/50$ HCl neutralised by the ammonia distilled over.

Arrows indicate commencement of frothing.

The same bulk of fluid was used for each curve. The ammonia content was the same for each curve.

If working with unknown solutions a time curve for the conditions of experiment will show immediately whether the results obtained are reliable. If the curve resembles that for 90 % alcohol, the conditions are correct. If the zero period is absent or very short it is due to too low a percentage of alcohol or too small a total volume of fluid. This can be remedied accordingly. Thus the great value of an initial time curve for any accurate work is evident.

THE ESTIMATION OF UREA.

This is done by converting the urea into ammonium carbonate by the action of a strong solution of urease. At 40° C. complete conversion was

invariably obtained in 20 minutes. The urease solution was buffered to p_H 7.02 by means of a solution consisting of suitable proportions of 3 molar caustic potash and molar acid potassium phosphate solutions.

The effect of such a buffered urease solution on the analysis was first investigated.

Exp. 8. Analysis of 100 cc. fluid containing 1 cc. ammonia and 9 cc. strong urease solution buffered to p_H 7.02.

Cc. alcohol	Ammonia present cc. <i>N</i> /50	Ammonia found cc. <i>N</i> /50
90	44.10	44.15
90	44.10	44.10
90	44.10	44.10

This experiment shows that urease has no effect upon the amount of ammonia distilled over up to frothing from 100 cc. of a 90 % alcohol solution of ammonia.

Solutions containing 25 % and 0.04 % of urea were next analysed and the results compared with those given by the aeration method.

Exp. 9. Analysis of 1 cc. of 25 % urea solution.

Correct result 80.10 cc. <i>N</i> /10. Results in cc. <i>N</i> /10.				
Method	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	80.05	80.10	80.10	80.08
Van Slyke aeration	80.10	80.15	80.15	80.13

Exp. 10. Analysis of 10 cc. of 0.04 % urea solution in 100 cc. 90 % alcohol.

Results in cc. <i>N</i> /50.				
Method	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	6.35	6.37	6.37	6.37
Van Slyke aeration	6.34	6.33	6.36	6.34

The method is therefore quite accurate for the estimation of urea in either strong or weak solutions.

APPLICATION TO URINE.

We then successfully attempted to apply the method to the analysis of urine.

In accordance with the recommendations made earlier in this paper our first experiment was to obtain a "time-ammonia" curve for the apparatus when 5 cc. normal urine, 90 cc. alcohol, and the equivalent in 5 cc. of ammonium carbonate solution of about 45 cc. of *N*/50 acid were present in the distilling flask. The curve obtained is shown in Fig. 3 c. The actual values are given below, *Exp. 16*.

The curve is of the type given by the 90 % alcohol solutions shown in Fig. 2. All the ammonia has therefore been distilled over before frothing commences. The conditions were therefore assumed to be correct and the following experiments were made.

A series of analyses of normal urine were made by this method and compared with the results given by the Van Slyke modification of the Folin aeration method.

Exp. 11. Analysis of 5 cc. urine in 100 cc. fluid containing 90 % alcohol.

Method	Results in cc. N/50.				
	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	7.25	7.20	7.24	7.25	7.235
Van Slyke aeration	7.22	7.24	7.25	7.25	7.240

Exp. 12. Analysis of 5 cc. urine in 100 cc. 90 % alcohol containing added ammonium carbonate.

Method	Results in cc. N/10.				Mean
	1st exp.	2nd exp.	3rd exp.		
Steam-alcohol distillation	44.12	44.10	44.15		44.120
Van Slyke aeration	44.10	44.15	44.10		44.120

This is as large a *quantity* of ammonia as is ever present in the quantities of urine which we recommend using, either before or after the urea has been converted into ammonium carbonate. The time curve (not shown) of this experiment was quite satisfactory.

These experiments show that the method is quite accurate for the estimation of ammonia in normal urine.

The urea was next estimated. 1 cc. of urine was acted on by 9 cc. of standard urease solution at 40° for 20 minutes. All the urea was found to be converted into ammonium carbonate by this procedure.

Exp. 13. Analysis of 1 cc. urine after treatment with urease to convert the urea into ammonium carbonate. 100 cc. of 90 % alcohol were used.

Method	Results in cc. N/50.				
	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	35.40	35.45	35.45	35.45	35.44
Van Slyke aeration	35.40	35.45	35.45	35.45	35.44

Again there is complete agreement with the Van Slyke-Folin method.

The method is therefore unaffected by urease in the presence of urine and it is accurate for the estimation of ammonia and urea in normal urine.

We then ascertained whether the method was equally accurate for the estimation of ammonia and urea in pathological urines. To do this we analysed samples of urine containing various foreign substances both with and without urease.

Exp. 14. Analysis of 5 cc. urine in 100 cc. 90 % alcohol in the presence of foreign substances.

Substance introduced	Results in cc. N/50.					
	Ammonia present	Ammonia found	Substance introduced	Ammonia present	Ammonia found	
1 g. glucose	7.00	7.00	1 cc. butyric acid	7.00	6.95	
"	7.00	7.00	1 cc. conc. butyric acid	7.00	7.00	
1 g. serum albumin	7.00	6.95	1 cc. 4 % aceto-acetic acid	7.00	7.00	
"	7.00	6.95	" "	7.00	7.00	
1 cc. conc. acetone	7.00	6.98				
"	7.00	6.95				

The results found in the presence of urease were exactly similar.

We conclude from these experiments that the method is applicable with great accuracy to the estimation of ammonia and urea in all urines whether normal or pathological.

Although the alcohol used may be easily recovered from the distillate after titration it is desirable that as little as possible be used when a large number of estimations are being made. We therefore investigated the effect of halving the volume of alcohol used, so that the total volume of fluid was only 50 cc. instead of 100 cc. We first repeated Exp. 11 using this smaller bulk.

Exp. 15. Analysis of urine in 50 cc. of fluid.

Cc. alcohol	Cc. urine	Ammonia present cc. N/50	Ammonia found cc. N/50
45	5	42.35	42.30
45	5	42.35	42.35
40	1 + 9 cc. urease	39.12	39.15
40	1 + 9 cc. „	39.12	39.10

For these values therefore the correct result is obtained with the smaller bulk.

From Exp. 3 it will be remembered, however, that 42.6 cc. N/10 ammonia (the maximum amount which can possibly be present in our urine estimations) is not quite all distilled over before frothing commences when a total bulk of 50 cc. of 90 % alcohol is used. The same holds good for urine and is an objection to the use of a smaller quantity of alcohol than 90 cc.

The time curves were then investigated and compared with those of equal percentage alcohol content but of double the volume of fluid.

The curves are shown in Fig. 3.

Exp. 16. Time curves for urine. See Fig. 3. The following table illustrates the nature of the data from which the curves were constructed. Frothing occurred at the time denoted by black figures.

Analysis of 5 cc. urine A in 100 cc. and B in 50 cc. 90 % alcohol. Ammonia content = 42.35 cc. N/50.

Time from commencement		Cc. N/50 ammonia distilled over	
A	B	A	B
m. s.	m. s.		
2 0	2 0	16.0	18.2
5 0	4 20	42.0	41.7
5 20	4 50	42.3	42.3
6 0	5 20	42.3	42.3
6 40	6 0	42.3	42.8
7 30	8 0	42.4	43.3
9 0	10 0	43.1	44.6
12 0	13 30	45.1	46.6
15 0	—	49.7	—

From this experiment it is clear that in the smaller bulk the zero period is smaller than in the larger bulk of equal alcohol percentage; and, bearing this in mind, there are two objections to using the smaller bulk.

(1) The zero period is shorter in the smaller bulk than in the larger bulk of equal alcohol concentration and therefore if the distillation is not stopped exactly at the commencement of frothing an error may be introduced.

(2) If the ammonia present is greater than 42.35 cc. $N/50$ all the ammonia may not have distilled over before frothing commences.

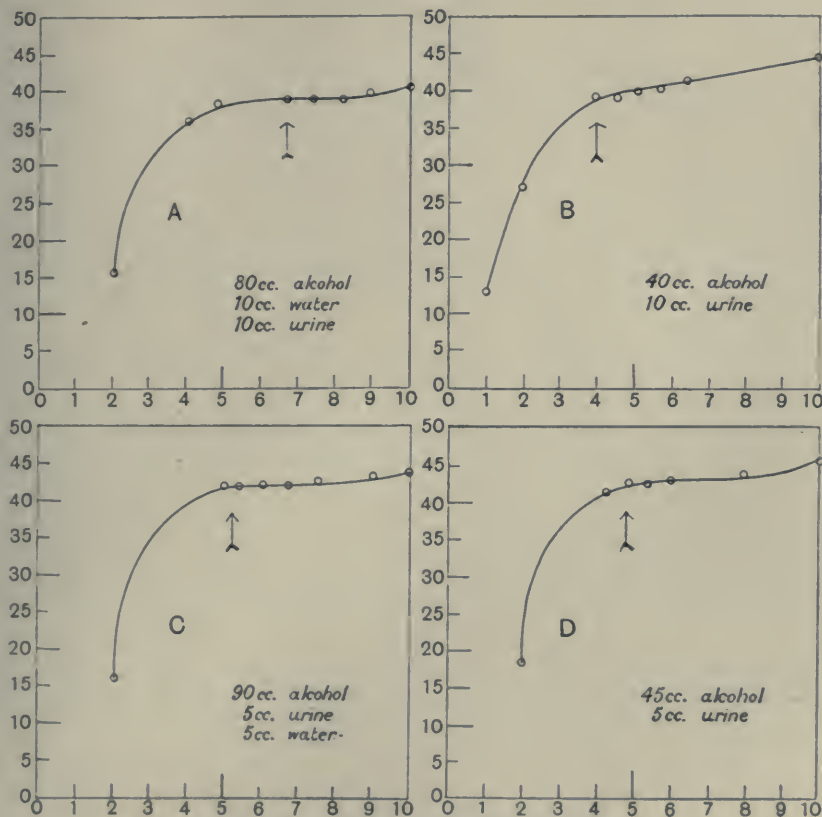


Fig. 3. Time curves for urines. See Exp. 16.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Abcissae = time in minutes from commencement of analysis.

Arrows indicate commencement of frothing.

The ammonia content was the same in all cases.

This error may be avoided, of course, by using smaller quantities of urine.

For general work therefore we recommend conducting the analysis in 100 cc. of 90 % alcohol which we have shown to give accurate results in all cases. For analysing quantities of ammonia less than 42.35 cc. $N/50$, however, a total volume of 50 cc. may be used with perfect accuracy.

SUMMARY AND CONCLUSION.

1. It is shown that the estimation of ammonia and urea by means of a steam-alcohol distillation is accurate to a high degree for all quantities of ammonia investigated, from 0.395 cc. *N*/50 to 144.1 cc. *N*/10 ammonium carbonate, which represents the analysis of a saturated solution of this salt. The small differences shown in some results are within the limits of error of the titration.

2. The conditions governing the steam alcohol distillation of ammonia were thoroughly investigated and it was found that when the analysis was conducted in 100 cc. of 90 % alcohol all the ammonia was distilled over before frothing commenced. This gave accurate results for all concentrations of ammonia up to saturation.

3. It is shown that the accuracy of the method in any particular case may be tested by obtaining a time analysis curve, the form of which indicates whether correct results are being obtained; although the ammonia content may not be otherwise known.

4. The method is proved to be accurate for the investigation of the action of urease on urea.

5. The method is rapid. A complete determination of ammonia and urea may be made with one apparatus in 30 minutes, including the time taken for the conversion of urea into ammonium carbonate. A determination of ammonia requires seven minutes only.

6. The method is applied successfully to the estimation of ammonia and urea in urines and is shown to hold good for pathological as well as for normal urines.

In our opinion the accuracy, rapidity, and facility of manipulation which the method possesses render it more desirable as a means of estimating ammonia and urea than any of the methods at present in use.

REFERENCE.

Foreman (1920). *Biochem. J.* 14, 451.

XLIV. NOTE ON THE ESTIMATION OF UREA BY UREASE.

By GEORGE MACFEAT WISHART.

From the Institute of Physiology, University of Glasgow.

(Received April 24th, 1923.)

IN the course of an investigation into the reason for a series of erroneous results obtained by the Soja bean-urease method for the estimation of urea, it was discovered that the estimation could be carried out much more rapidly and easily by doing the hydrolysis of the urea and the evacuation of the formed ammonia simultaneously. Curiously enough, if the bean be used in its crude powdered state, it is possible to add to the urea-containing solution the requisite quantity of sodium carbonate to liberate the formed ammonia without interfering with the hydrolytic action of the urease; the alkali would appear to be taken up by some constituent of the bean, possibly the proteins, and thus prevented from exerting its inhibitory action on the enzyme.

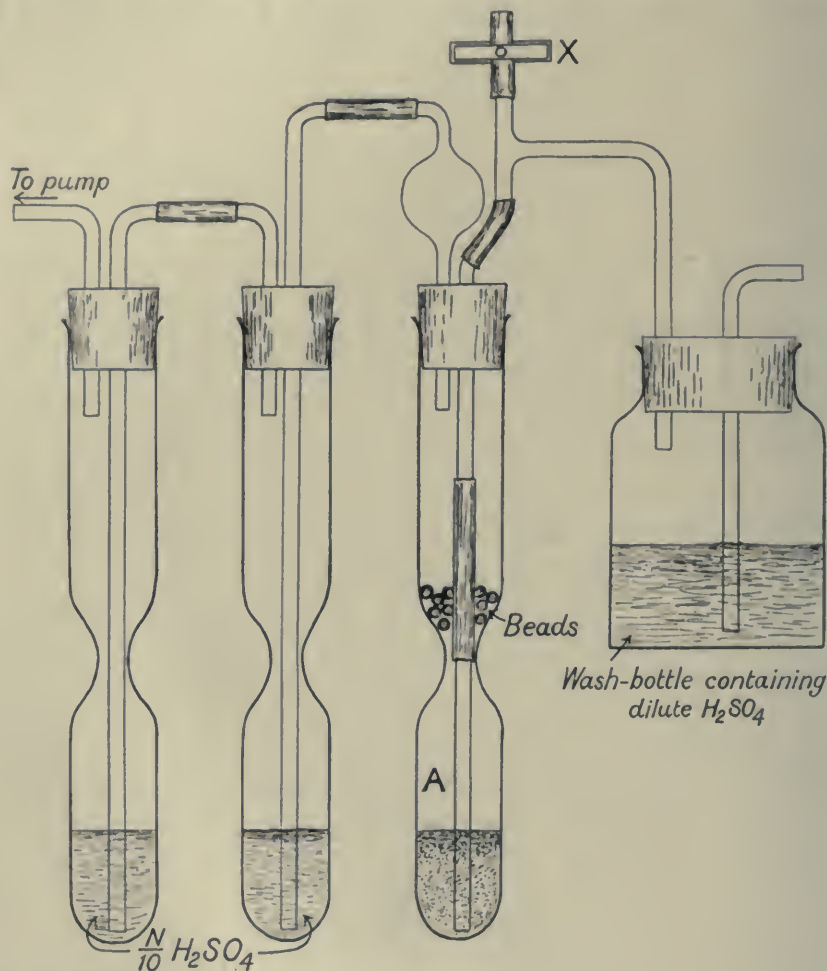
A large number of experiments were performed in which the vessel containing the $N/10$ H_2SO_4 for absorbing the liberated ammonia was connected with a burette, and thus the course of the evolution of the ammonia could be followed, minute by minute. Such experiments served for a comparison of various types of air-inlet tubes, and for the determination of the optimum temperature, and the optimum quantities of the reagents. In the course of these experiments it was found that the more rapid the aeration, the more rapidly could the estimation be completed; *e.g.* in one experiment with an air-current of 6 litres per minute, 75 % of the urea-ammonia had been liberated in 10 minutes, while with an air-current of 10 litres per minute, 92.4 % of the theoretical amount was liberated within the same time.

To provide for as rapid an air-current as possible, and at the same time to prevent loss of ammonia, and frothing of the solutions, the apparatus was modified as shown in the diagram.

Tube *A* and the absorbers are made from the tubes of an ordinary ammonia absorption apparatus. The clip at *X* allows of the saturated sodium carbonate solution being introduced after the whole apparatus is connected up and the air-current started, without any disjoining of the tubing, thus making loss of ammonia impossible.

The constrictions in all three tubes should be placed at such a level that the capacity of the lower bulb is about three times the volume of fluid to be placed within it. This arrangement, along with the use of glass beads in tube *A*, renders the use of any anti-frothing solution unnecessary in most cases.

To complete the estimation in the times given, the pump should be capable of drawing air through the apparatus at a rate of not less than $5\frac{1}{2}$ litres per minute. The water-bath temperature should be maintained at about 40° .



Method of Estimation.

In tube *A* place 20 cc. water, 5 cc. urine, and 3–5 g. Soja bean meal (varying amounts are required according to the condition of the bean; a slight excess will not vitiate the result). Mix the contents of the tube and insert the central tube and glass beads as shown, immerse bulb in water-bath, then connect up to collecting tubes containing a known amount of decinormal acid. Start the pump and introduce about 2 cc. of a saturated solution of sodium carbonate from a pipette by holding the nozzle of the pipette at *X* and opening the clip. Close the clip and aerate for 40 minutes.

A smaller apparatus with tubes of about 100 cc. capacity, and using $N/50$ acid, has been found useful for estimating small amounts of urea. The quantities for this apparatus are: 5 cc. of urea-containing fluid (content not more than 10 mgm. urea); 1 g. bean meal; and 0.5 cc. saturated sodium carbonate solution. Aeration with this smaller apparatus, and using these quantities, is complete in 20 minutes.

Since the time required for complete evacuation of the ammonia depends on the velocity of the air-current and the amount of bean meal used, it is well to determine that all conditions are correct by doing one estimation on a solution containing a known amount of pure urea. The bean meal liberates a certain small amount of ammonia itself, so that, for accurate work, it is necessary to estimate this by doing a blank experiment, using water in place of the urea solution.

As examples of the results obtained by this method the following may be given.

Large apparatus (N/50 acid and alkali used):

Experiment	Theoretical amount	Estimated amount
1	1 %	0.9948 %
2	1	0.9936
3	1	0.9912

Small apparatus:

1	0.004 %	0.00408 %
2	0.004	0.00396
3	0.004	0.00402

Experiments on the estimation of urea in urines with and without added quantities of urea gave equally satisfactory results. In estimating urea in urine by the larger apparatus, it is usually advisable to dilute the urine so that it will contain approximately 1 % of urea.

XLV. THE ACCURACY OF THE DALE AND EVANS METHOD OF DETERMINING THE HYDROGEN ION CONCENTRATION OF BLOOD.

BY HAROLD TAYLOR.

From the Physiological Laboratory, Manchester.

(Received April 27th, 1923.)

THE method employed by Dale and Evans [1920] of finding the hydrogen ion concentration of blood is to place the blood in a small thin collodion tube and to suspend this in an isotonic saline solution. The whole is allowed to stand for about half-an-hour to allow equilibrium to be attained, and the hydrogen ion concentration of the outside solution is then determined by an indicator method. The hydrogen ion concentration of this solution is then taken as that of the blood. The hydrogen ion concentration determined by this method does not, however, according to Evans [1920] agree with the results obtained by the use of a hydrogen electrode placed in the blood, the hydrogen electrode giving, in his hands, results which were about $0.2 p_H$ smaller than those obtained by the dialysis method. The diffusion method is supposed to be accurate to $0.02 p_H$; at any rate the p_H of the dialysate can be determined to that degree of accuracy. Since the phosphate solutions used for comparison with the dialysate are standardised with a hydrogen electrode, it is quite clear that the p_H of the dialysate must be approximately correct, and hence the difference must be due, either to the presence of the collodion membrane or to some effect of the blood on the electrode.

Evans in his paper suggests a number of possible causes to account for the difference observed, but he does not consider the fact that even in equilibrium the concentration of the hydrogen ions may not be the same on both sides of the membrane. Such would be the case if a Donnan equilibrium were set up at the membrane. Considering the proteins of blood as providing ions incapable of diffusing through the collodion membrane we have the conditions necessary for the existence of a Donnan equilibrium, *i.e.* on one side of the membrane an indiffusible ion, along with diffusible ions, and on the other side diffusible ions only. Thus when equilibrium is set up there will not be an equal distribution of diffusible ions on the two sides of the membrane. Such a state has actually been demonstrated by Loeb [1921] in the case of

gelatin solution inside a collodion tube, the concentrations of the hydrogen ions being unequal on the two sides of the membrane.

If two liquids on opposite sides of a membrane be in equilibrium and the concentrations C_1 and C_2 of the diffusible ions be not the same on both sides, then there must be a potential difference across the membrane. This potential difference has been demonstrated by Loeb [1921] and whatever be the cause of the inequality of concentration it can be shown thermodynamically [Hill, 1923] that the potential difference E must be of the form

$$E = \frac{R \cdot T}{n \cdot F} \log \frac{C_1}{C_2}.$$

[n = valency of ion; F = electrical charge per equivalent.]

Hence by measuring the potential difference between the blood inside and the saline solution outside the ratio of the concentration of hydrogen ions in the blood to that in the outer solution can be determined. In the case of hydrogen ions at 18° the formula reduces to $E = 59 [p_{\text{H}} (\text{inside}) - p_{\text{H}} (\text{outside})]$ millivolts. It can be shown also that, in the case of a membrane potential, the side on which the concentration of hydrogen ions is the lower is the positive side.

In the presence of a considerable concentration of diffusible ions the difference of concentration occurring in a Donnan equilibrium must be small. In the case of blood therefore no considerable potential difference can be expected.

Method of measurement of membrane potential.

The potential difference across the membrane was measured by means of a potentiometer and two saturated KCl calomel electrodes. In many of the observations a high resistance galvanometer of extreme sensitivity was employed. The apparatus was the same as used in the actual determination of the hydrogen ion concentration by the Dale and Evans method. The small tube into which the saline was put was located in a wider tube, which was large enough to hold the whole of the apparatus when the vulcanite top had been placed on. The amount of saline in the small glass tube was arranged so that when the dialysing tube containing the blood was placed in it the saline came up to the top of the glass tube. When the whole was ready it was corked up and left for about three-quarters of an hour to ensure that equilibrium was attained. This is longer than the time generally allowed in hydrogen ion determinations. The outer tube was then filled with saturated KCl solution up to the lower rim of the vulcanite top. The cork of the dialysing tube was then removed, one saturated KCl calomel electrode placed in the blood and the other in the outer KCl solution. The calomel electrodes made contact through capillary points so as to avoid contaminating the blood with KCl. The potential difference was then measured between the two electrodes. Enough electrical contact was obtained round the edge of the vulcanite top, between

the dialysate and the KCl outside, for the readings to be made easily. The cell employed therefore was as follows:

Calomel electrode	Saturated KCl	Blood	Membrane	0.85 % saline	Saturated KCl	Calomel electrode
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Due allowance was made for any difference between the calomel electrodes employed by testing them together in the same solution. The results are given in the following table.

No. of experiment ...	1	2	3	4	5	6
p.D. (millivolts) }						
Blood positive }	2.44	2.90	2.31	0	-3.45	+1.15
p_{H} (inside) }						
$-p_{\text{H}}$ (outside) }	0.041	0.049	0.039	0	-0.058	0.020
c_{H} inside						
c_{H} outside	0.91	0.83	0.91	1.0	1.14	0.95
No. of experiment ...	7	8	9	10	11	12
p.D. (millivolts) }						
Blood positive }	-5.3	-0.9	3.23	0.1	0.85	-0.5
p_{H} (inside) }						
$-p_{\text{H}}$ (outside) }	-0.090	-0.015	0.055	0.002	0.018	-0.009
c_{H} inside						
c_{H} outside	1.22	1.035	0.88	0.99	0.96	1.02
No. of experiment ...	13	14	15	16	17	18
p.D. (millivolts) }						
Blood positive }	2.2	1.3	0.76	4.42	2.54	1.66
p_{H} (inside) }						
$-p_{\text{H}}$ (outside) }	0.037	0.022	0.013	0.075	0.043	0.028
c_{H} inside						
c_{H} outside	0.92	0.95	0.97	0.86	0.91	0.94
No. of experiment ...	19	20	21	22		
p.D. (millivolts) }						
Blood positive }	0.45	0.66	0.53	0.75		
p_{H} (inside) }						
$-p_{\text{H}}$ (outside) }	0.008	0.011	0.009	0.013		
c_{H} inside						
c_{H} outside	0.98	0.97	0.97	0.97		

In the above table, in spite of small individual variations, the ratio of c_{H} (inside) to c_{H} (outside) is consistently about unity. Its average value is 0.97, the average deviation from this value being only 0.06 (about 6 %), the probable error of the mean value only about 0.01 (about 1 %). A ratio of 0.97 between the true c_{H} of the blood and that of the dialysate corresponds to a p_{H} difference of only 0.013, which is outside the limits of accuracy of measurements by the dialysis method. The average deviation in the table corresponds to a p_{H} difference of 0.027 which is of the same size as the admitted error of the dialysis method. Hence, for practical purposes we may regard the method of Dale and Evans as giving correctly the hydrogen ion concentration of the blood as existing at the moment when the dialysis has been completed: for greater exactness 3 % may be subtracted from the c_{H} or 0.013 added to the p_{H} , to allow for the membrane effect.

If the membranes are used several times for determinations of the hydrogen ion concentration, they become stained and in such cases potential differences up to 11 millivolts, are obtained. It was found, however, that if 0.85 % saline

was placed on both sides of these membranes a potential difference was set up across them of the same order of size as obtained with blood on one side. Hence the stained membranes, for some unknown reason, introduce a large error into the hydrogen ion determinations. The membranes therefore were always tested before use by finding the membrane potential with saline on both sides. In most cases no potential difference was obtained but a small P.D. was found when the membranes had become slightly stained while testing for leaks. Thus for accurate determinations of the hydrogen ion concentration new membranes should be employed, or at any rate such as have been used before should be carefully tested before being used again.

SUMMARY.

In the Dale-Evans method of determining the hydrogen ion concentration of blood it was conceivable that an appreciable error might occur owing to a difference of concentration (due for example to a Donnan equilibrium) produced by the membrane itself. Such an effect would result in a measurable electrical potential difference. The actual P.D. has been measured and found to be small. On the average the hydrogen ion concentration in the blood is only 3 % less than in the dialysate, an amount smaller than the error of the method. For practical purposes, therefore, the c_{H} of the dialysate is identical with that of the blood as existing at the moment when dialysis is complete.

I wish to express my indebtedness to Dr R. E. Conway for the provision of the Dale-Evans apparatus, and for valuable suggestions.

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XLVI. THE INFLUENCE OF REACTION ON THE OXIDATION OF THE ANTISCORBUTIC FACTOR IN LEMON JUICE.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received May 7th, 1923.)

THE earliest investigations of experimental scurvy have revealed that reaction influenced the stability of the antiscorbutic factor. Holst and Frölich [1912] were the first to draw attention to the fact that in acid solution the antiscorbutic potency displays greater stability than in alkaline solution. This observation has since been confirmed by various workers [Harden and Zilva, 1918; Hess and Unger, 1919; La Mer, 1921]. Further work on the stability of the antiscorbutic factor has shown that oxidation has also a destructive action on the potency of active solutions [Hess and Unger, 1921; Zilva, 1921; Dutcher, Harshaw and Hall, 1921].

This paper deals with the elucidation and correlation of the inactivating influences of reaction and oxidation. The experiments were instituted as a result of some suggestive information obtained in connection with another inquiry on adsorption, which is still in progress. Evidence is here produced which shows that in the case of decitrated lemon juice the destruction of the factor is a process of oxidation which proceeds rapidly in alkaline solutions and slowly in an acid medium. Under anaerobic conditions alkaline reaction seems to have no inactivating influence; in presence of air when the hydrogen ion concentration is raised the destruction of the antiscorbutic potency is delayed. An active juice made approximately $N/20$ alkaline (p_H 12.5) and kept at this hydrogen-ion concentration for 24 hours under strictly anaerobic conditions did not deteriorate to a marked extent, whilst the same juice and of the same reaction kept exposed to the air during the same period failed to protect or even to delay the onset of scurvy in guinea-pigs in a dose about 5-6 times larger than that which protected the animals from the disease when the juice was kept anaerobically. Furthermore, when the hydrogen-ion concentration was brought up to p_H 2.2 the inactivation by aeration at 100° was definitely delayed.

At p_H 12.5, in the presence of air and at room temperature, the destruction of the antiscorbutic factor proceeds fairly rapidly. After three hours it is almost completely destroyed whilst after half an hour the activity is diminished to a considerable extent.

EXPERIMENTAL.

All the preparations were tested out on guinea-pigs. The animals were kept on a scorbutic diet of oats and bran supplemented by a daily ration of not more than 40 cc. autoclaved milk. On this diet guinea-pigs develop scurvy in about 10–15 days and succumb to the disease after about four weeks. On the other hand when this diet is supplemented by an adequate antiscorbutic dose the animals thrive well on it.

The doses to be tested were administered daily *per os* after the animals had been on the scorbutic diet for about 14 days. In the case of a positive result the duration of the experiment was two months, after which time the guinea-pigs were chloroformed and submitted to a post mortem examination.

The Influence of Alkalinity on the Antiscorbutic Factor under Aerobic and Anaerobic Conditions.

The citric acid was removed from the lemon juice by the addition of excess of calcium carbonate and two volumes of absolute alcohol. After filtration, the solution was concentrated in a vacuum at 40–50° and eventually brought up to the original volume of the juice. The reaction was then adjusted colorimetrically to p_H 6.6–6.8. In the aerobic experiments this adjusted juice was made approximately $N/20$ by the addition of normal sodium hydroxide (1.3 cc. of N NaOH to 23.7 cc. of juice) and allowed to stand in a conical flask in the laboratory for 24 hours before being administered to the experimental animals. Great precautions were taken in the anaerobic experiments to exclude air. The adjusted decitrated juice was introduced into bottles of small capacity (about 50 cc.) immediately after the addition of the requisite alkali for the production of a $N/20$ alkaline solution, the bottle was closed with a tight-fitting rubber stopper containing a glass tube with a tap and rapidly exhausted by means of an air pump. The bottle was then placed under a bell-jar over alkaline pyrogalllic acid which was in its turn exhausted. The hydrogen-ion concentration of samples of decitrated juice made alkaline was determined electrometrically both immediately after the addition of the alkali and after storage under anaerobic conditions. No significant difference could be detected in the reaction. The actual figures found in one of these cases were as follows:—immediately after the addition of alkali $p_H = 12.12$, the same juice kept under anaerobic conditions for 24 hours $p_H = 12.09$. When, however, the juice was made alkaline and allowed to stand exposed to the air the hydrogen-ion concentration gradually increased. I should like to take this opportunity of thanking Dr E. E. Atkin for having kindly carried out the electrometric estimations.

At the expiration of 24 hours the aerobic and anaerobic solutions were acidified and fed to the animals. These preparations were made daily. Doses of 1.5, 3, 5 and 7 cc. were given of each preparation. The aerobic alkaline solutions were found quite inactive, whilst the same alkaline solutions kept

anaerobically were found to be protective even in the lowest dose of 1.5 cc. Fig. 1 gives the weight curve of the animals receiving the highest dose.

It was further of interest to ascertain the time taken to bring about the total inactivation of the antiscorbutic factor in a solution of the above alkalinity when kept exposed to the air. For this purpose the decitrated lemon juice was made approximately $N/20$ alkaline as before and kept in small open conical flasks at room temperature for 0.5 hour, 1 hour and 3 hours. After the respective times of treatment, the solutions were acidified by the addition of citric acid (0.1 g. to 10 cc.) and administered to the animals the same day. The tests have disclosed that after keeping the decitrated lemon

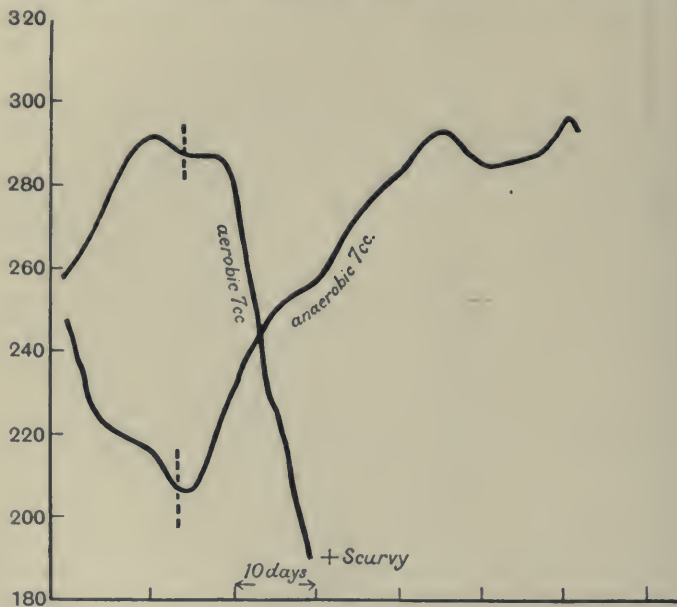


Fig. 1.

juice alkaline for one hour a daily dose of 5 cc. showed no potency. The same dose of decitrated lemon juice kept alkaline for 0.5 hour was found to be active as will be seen from Fig. 2 although the animal receiving this dose was found to show signs of incipient scurvy when chloroformed after two months. Doses of 1.5 cc. and 3 cc. of the same preparation were however ineffective to prevent or even to delay the onset of scurvy. As the minimum protective dose of decitrated lemon juice is 1-1.5 cc., it seems that about 80 % of the antiscorbutic factor has been destroyed in half an hour.

The Influence of Acidity on the Inactivation of the Antiscorbutic Factor by Aeration at 100° C.

In these experiments the lemon juice was prepared as in the previous experiments and adjusted to p_{H} 6.6-6.8. Some of the juice was brought to p_{H} 2.2-2.4 by the addition of citric acid. Both preparations were boiled under

a reflux condenser, air being aspirated through the boiling liquid. The decitrated lemon juice of p_{H} 6.6-6.8 was treated in this way for one hour. The acidified juice was aerated for one hour and for two hours.

Daily doses of 1.5, 3 and 5 cc. of the three preparations were tested out on animals. In all the three preparations 1.5 cc. of the solution failed to show any protective properties, the higher dose of 3 cc. failed to protect in the neutral preparation and in the acid solution aerated for two hours. This dose of the acid preparation which was aerated for one hour offered partial protection—the animal surviving for two months although it was in a declining and scorbutic condition at the end of that period. The difference in the degree of inactivation was best evident in the experiment in which the 5 cc. daily doses



Fig. 2.

were used. Fig. 3 gives a graphic presentation of the weights of the animals which were given 5 cc. daily of each of the preparations. The animal which received the acid preparation aerated for one hour was chloroformed after two months and although some mild signs of scurvy were established it was nevertheless evident that the guinea-pig could have lived for some time longer. The animal which received the same dose of neutral preparation aerated for one hour commenced declining slowly after 15 days and died after 40 days from acute scurvy. Doubtful protection was achieved by 5 cc. of acid preparation aerated for two hours. These experiments show that the acidity undoubtedly had a protective action on the inactivation of the antiscorbutic factor by aeration at 100°.

Decitrated lemon juice as prepared above contains 0.8 %–1 % of solids, the best part of which consists of sugars. It is, therefore, reasonable to assume that the increase in the hydrogen-ion concentration when the alkaline juice is exposed to the air is due to the production of acids from the sugar, which according

to the extensive researches of Nef is oxidised in alkaline solution to various organic acids. Although there is a certain parallelism between the formation of acid and the inactivation of the antiscorbutic factor in the experiments described, in so far that both take place in alkaline solution in the presence but not in the absence of air, the parallelism is not complete. It is seen that about 80 % of the factor is destroyed by the treatment in about half an hour. Preliminary investigations show that the formation of acids proceeds at a much slower rate. After half an hour the hydrogen-ion concentration is only

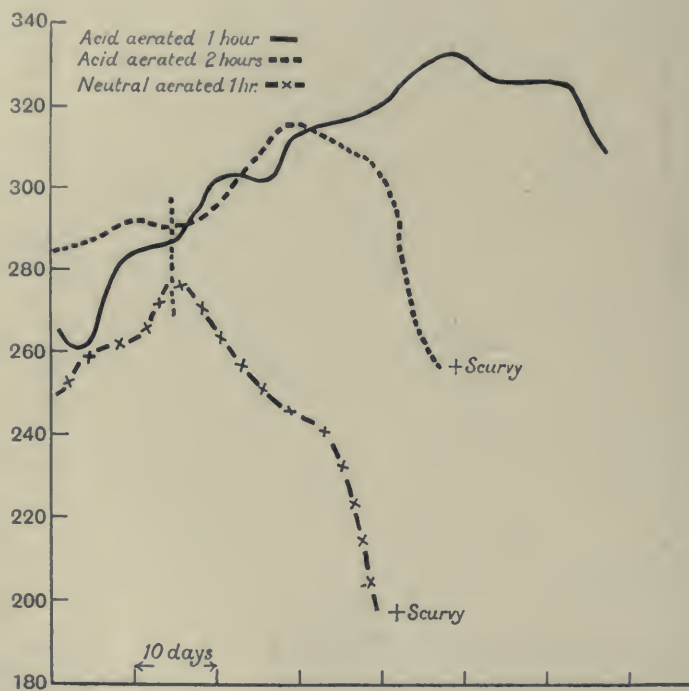


Fig. 3.

just perceptibly increased. Whether the inactivation of the antiscorbutic factor is due to an intermediate change of the sugar before the formation of the acid or whether it is a change in which the sugar is not concerned cannot be answered from the experimental evidence so far available. The writer hopes that investigations on the subject which are now in progress may conduce to the elucidation of this problem.

SUMMARY.

Decitrated lemon juice made approximately $N/20$ alkaline ($p_{11} = 12.5$) and kept exposed to the air at room temperature loses about 80 % of its antiscorbutic potency in half an hour. After three hours a daily dose of 5 cc. fails to prevent or delay the onset of scurvy in guinea-pigs.

Decitrated lemon juice of the same reaction kept for 24 hours at room temperature in the absence of air shows no deterioration in its antiscorbutic potency.

There is no change in the hydrogen-ion concentration of the alkaline decitrated juice kept in the absence of air after 24 hours; when, however, it is exposed to the air there is an increase in the hydrogen-ion concentration.

When air is aspirated through a boiling solution of decitrated lemon juice for one hour the destruction of the antiscorbutic factor is delayed when the hydrogen-ion concentration is raised from p_{H} 6.6 to 2.2.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

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XLVII. A NOTE ON THE CONSERVATION OF THE POTENCY OF CONCENTRATED ANTISCORBUTIC PREPARATIONS.

BY SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

(Received May 14th, 1923.)

SOME years ago it was shown that decitrated lemon juice prepared in concentrated form was of therapeutic value in the treatment of infantile scurvy [Harden, Zilva and Still, 1919]. The advantage of such a treatment is that very potent antiscorbutic solutions can be administered in very small bulk without introducing at the same time most of the extraneous matter present in active juices derived from natural products. During the last few years the writer had had the opportunity of preparing on a number of occasions concentrated decitrated lemon juice solutions which were used with great success in cases of acute infantile scurvy. These preparations were found to be especially of great value when the patients were suffering from gastric disturbances which made the administration of ordinary antiscorbutics difficult. As an example of this one may quote a recent case of Dr G. F. Still at the Hospital for Sick Children, Great Ormond Street, to whom I am indebted for the following particulars.

Patrick M..... Age 10/12. Admitted Jan. 29, 1923.

Tenderness of limbs since November. Fed on Allenbury foods, No. 1 till 4 months, No. 2 till 7 months, No. 3 (made with milk) until December when he was put back during the last 2 or 3 weeks to No. 2. Has also had some oatmeal water but nothing else.

On admission did not move arms or legs. Screamed with pain when arms or legs touched, especially wrists and ankles; some thickening just above ankles and wrists; apparently periosteal haemorrhage: haemorrhages into gums around incisors; urine, no blood; temperature varying up to 103.4° F.

Treated with potato cream and orange juice; one teaspoonful of each twice a day.

After 6 days, child more comfortable but temperature irregular.

Orange juice given four times daily. By February 5th stools became frequent, green, and loose, and from this time, although the child became much less tender, the looseness of the bowels, with much mucus, continued; there was no naked eye blood in the stools.

By February 19th the child was looking very bad; temperature 104°, eyes sunken, and looked as if likely to die. The ordinary antiscorbutics had been stopped on account of the bowel condition.

Concentrated decitrated lemon juice begun on February 21st; temperature reached normal on February 22nd, rose to 101° F. on 23rd and then fell to normal or thereabouts; the stools steadily improved.

The concentrated lemon juice was continued until March 5th giii per diem, except from February 21-25 when $\frac{1}{2}$ oz. was given daily; on the first day of an eight times concentrated solution (of which 2 oz. = 15 lemons), and on the second day a ten times concentrated solution, $\frac{1}{2}$ oz. was given.

The child continued to do well and gained weight during the next four weeks during which the periosteal thickenings gradually diminished until they had almost gone altogether.

No further trouble occurred, except that there was a gastro-intestinal disturbance a few days before the child was discharged from the Hospital on April 12th, but this did not appear to be connected in any way with his scurvy.

Such concentrated decitrated solutions, however, deteriorate in potency on keeping and consequently must be freshly prepared before administration—a circumstance which militates against their general use in medical practice. In view of the observations made by the writer on the inactivation of the antiscorbutic factor, conditions have been worked out which make it possible to store such concentrated antiscorbutic solutions without detriment to their activity. As it was found [Zilva, 1923] that destruction of the antiscorbutic factor is favoured by alkalinity and exposure to air, the concentrated antiscorbutic solutions were acidified and stored under anaerobic conditions. A concentrated juice stored in this way did not show any perceptible loss in potency after storage for three months.

Two and a half litres of lemon juice were treated with an excess of calcium carbonate and three volumes of absolute alcohol and filtered. The filtrate was concentrated to a tenth of its volume and acidified slightly. It was then stored in an ordinary conical flask in an evacuated bell-jar over alkaline pyrogallic acid for three months. At the commencement of the experiment a suitable quantity of the concentrated juice was diluted to its original volume and tested out on guinea pigs in order to determine its minimum protective dose. During the period of testing (about six weeks) the diluted juice was also kept under an evacuated bell-jar over alkaline pyrogallic acid, the necessary dose being removed daily. The concentrated and diluted preparations were kept at ordinary room temperature. At the end of three months another quantity of the concentrated preparation was diluted and tested out in the same way as at the commencement. As anticipated no loss of potency was observed. In both cases a daily dose of 1.5 cc. of the preparation which was previously diluted to its original volume afforded protection, whilst daily doses of 0.5 cc. and 1 cc. of both the original and stored preparation only protected the animal partially, the onset of the disease and death being delayed.

Adopting the above principle a method is now being worked out for the preparation of stable concentrated antiscorbutic solutions which could find practical application in the prophylaxis and treatment of infantile and adult scurvy and which it is hoped will be of use to the clinician.

The expenses of this research were defrayed from a grant made by the Medical Research Council to whom my thanks are due.

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XLVIII. ARTIFICIAL PARTHENO-GENESIS IN SEA URCHINS.

A NOTE ON AN IMPROVED TECHNIQUE.

BY ISAAC FROST.

From the Department of Physiology, University of Birmingham.

(Received May 4th, 1923.)

WORKING at the Marine Biological Laboratory, Plymouth, at Easter, 1921, the writer followed Shearer and Lloyd [1913] in their modification of Loeb's [1913] technique for the induction of artificial partheno-genesis in sea urchin (*Echinus esculentus*) eggs.

The eggs were shaken into a bowl of fresh "outside" sea water and, if found of the right maturity, were transferred in turn by pipetting into a series of bowls containing the following solutions in the order given, and for the times stated:

- (1) 0.3 cc. $N/10$ butyric acid + 50 cc. sea water. 1.5 minutes.
- (2) Sea water. Wash
- (3) " " " } 15 minutes.
- (4) 0.2 cc. $N/10$ NaOH + 50 cc. sea water. 6 minutes.
- (5) Sea water. Wash.
- (6) 8.0 cc. 2.5 M NaCl + 50 cc. sea water. 45-60 minutes; or 25-30 minutes.
- (7) Sea water. Wash.
- (8) Final transfer into normal sea water.

These manipulations certainly resulted in segmentation of the eggs, and in the production of swimming blastulae. But the yield varied enormously from experiment to experiment; and, where it was necessary for other purposes to look for mass production, the above method, at any rate in the writer's hands, failed to give consistently good results. Conditions inherent in the egg could not account for the differences. For, specimens of eggs from the same batch treated in the same way at the same time displayed widely differing mortality. Plainly, the discrepancy crept in from outside. Errors in the composition of the various fluids would offer a sufficient explanation.

The pipette method of transfer seemed the most likely source of such error. At each transfer at least 50 %—more often 90 %—of the eggs remain behind in the bowl. On the former generous scale of diminishing returns, where there are at least eight transfers, the last bowl will contain $\frac{1}{2^8}$ ($\frac{1}{256}$ th) the number of eggs treated in the first bowl. To obtain any visible number of eggs in the end bowl, it was found that at least 3.0 cc. of sea water containing a thick emulsion of eggs had to be used to commence with.

Now, of what use is it to prepare, meticulously, the exactly proportioned solution containing *e.g.* 0.2 cc. N/10 NaOH in 50.0 cc. sea water when an immediate contamination of 3.0 cc., that is 6.0 % is involved in the mere transfer? More often, however, 6.0 cc. or even 9.0 cc. (18 % error) is transferred, or else it is quite impossible to pick out the eggs in the eighth bowl. One has to search for the number of eggs in $\frac{1}{250}$ of 3.0 cc. scattered in a solution of 50.0 cc. sea water. Plainly, at least 6 % error, and more often 12 % to 18 % error, is involved in each transfer. Here then, we may say we have discovered the source of the differing end-results.

The remedy is simple. The experiment must be so arranged that the eggs remain in the same vessel throughout; the changes of medium being effected by drawing off the fluid.

A deep funnel, with a securely fitted filter answers the purpose. The writer adapted the funnel of a Cona coffee still; the filter pad is firmly applied to the bottom of the funnel by means of a long wire passing through the limb of the funnel. A screw at the bottom of the wire serves to tighten the filter pad as much as desired.

The filter pad itself consists of a perforated metal or porcelain disc covered with several layers of fine muslin sewn into the disc. Layers of fine filter paper may be super-added if it is found that the eggs pass through.

A piece of rubber tubing, supplied with a tap, is fitted to the end of the limb, and in this way it is possible to permit the action of a solution for any length of time before withdrawing. Draining off can be effected much more rapidly by the judicious and gentle use of a vacuum water pump, to which the apparatus can be easily attached by rubber tubing. A further advantage in this method is that the eggs can be easily washed any number of times by flushing the apparatus through with sea water.

In conclusion, it may be said that the method is suitable not only for the partheno-genetic treatment of eggs, but for all experiments in which eggs, fertilised or unfertilised, are to be exposed to the action of various fluids.

SUMMARY.

(1) The "pipette" method of transferring eggs from one bowl to another is laborious, time consuming and involves a minimum error of 6 %; more often 12 %-18 %.

(2) The new "funnel" method described is easily adapted to laboratory material, involving the use of a flat-bottomed funnel with perforated plate, muslin and filter paper. A ready made efficient substitute is indicated in the funnel of the "Cona" coffee brewing apparatus.

(3) The method obviates the errors of transfer; it is simple, rapid and involves no eye-strain.

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XLIX. A SIMPLIFIED METHOD OF PREPARATION OF THE BEZSSONOFF REAGENT FOR VITAMIN C AND SOME POLYPHENOLS.

BY NIKOLAI BEZSSONOFF.

From the Laboratoire de Chimie Biologique de Colombes, France.

(Received May 18th, 1923.)

IN 1921, I described [1921] a blue coloration common to antiscorbutic extracts, to quinol and to catechol and obtained in acid medium with a phosphomolybdotungstic acid having the formula: $17 \text{ WO}_3 (\text{MoO}_3) (\text{P}_2\text{O}_5) 25 \text{ H}_2\text{O}$.

Concerning the reaction of this acid in the presence of antiscorbutic extracts, I noted the absence of a blue coloration with plum juice which is a juice devoid of any antiscorbutic action and on the contrary the presence of this coloration with the peach, though belonging to the same genus as the plum. I therefore concluded that the peach contained the antiscorbutic, although this fruit had not at that time been examined by animal tests. Since then Delf [1922] has verified the antiscorbutic action of the peach.

It seems then that this reagent is of value in the study of the antiscorbutic value of plants which have not been examined in the usual way.

It could equally serve to reveal the presence of polyphenols in different biological liquids (urines, etc.).

It seems useful to indicate a quick and simple method in order to obtain this reagent in a crystalline state and sufficiently pure: 36 g. of sodium tungstate and 4 g. of phosphomolybdic acid are dissolved in 200 cc. of distilled water at a temperature of about 50° ; then 5 cc. of 85 % phosphoric acid are added and 10 cc. of concentrated sulphuric acid are poured drop by drop into the above solution which should be stirred as the same time. The solution is evaporated slowly at a temperature of $40-42^\circ$. Slowly pale yellow monoclinic crystals are formed at the bottom and on the sides of the vessel. At the end of 20 to 24 hours, the solution being concentrated to about $1/3$, the liquid is poured off. In order to obtain a sufficiently pure reagent without proceeding to a new crystallisation, 2 to 3 cc. of distilled water are poured on the crystals. They are then stirred slowly and the liquid is immediately poured off. This operation is repeated until one drop of the water used for washing the crystals gives a blue coloration with a quinol solution or a brown-yellow with a pyrogallol solution (1 in 1000 in each case). The washed crystals are dried between filter paper, then 15 g. are dissolved in 100 cc. of 5 % (by volume) sulphuric acid.

Prepared in this way, the reagent can be preserved two months and more in coloured glass bottles using a ground glass or rubber stopper (avoid a cork stopper).

I recall [Bezssonoff, 1921, 1922] that this reagent must be employed in an acid medium. The sulphuric acid present in the solution generally suffices to secure this condition.

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L. STUDIES ON INOSITOL.

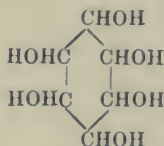
I. A METHOD OF QUANTITATIVE ESTIMATION.

BY JOSEPH NEEDHAM (*Benn Levy Student*).

From the Biochemical Laboratory, Cambridge University.

(*Received May 13th, 1923.*)

INOSITOL was first discovered in 1850 by Scherer [1850], who isolated it from Liebig's extract of meat and accurately determined its empirical formula. Between that date and 1864 it was studied by Cloetta [1856], Müller, W. [1857], Cooper-Lane [1861] and Marmé [1865] who gradually outlined the main features of the problem of its significance, ascertaining its distribution in plants and animals, and elaborating tests and methods of extraction. All the earlier workers regarded it as in constitution allied to the sugars, because of its empirical formula, $C_6H_{12}O_6$; and it was accordingly named "muscle-sugar," from one of its principal sources. Thus it was left for Maquenne to show in 1887 that inositol was not a sugar but a cyclic hexamethylene compound, or hexahydroxyhydrobenzene.



Ever since then, the obvious physiological implications of such a structure have attracted considerable interest, and the more so since the discovery of phytin, the calcium magnesium salt of inositolhexaphosphoric acid, in plants, by Palladin [1895], Winterstein [1897], Posternak [1903], and others. Throughout both periods all investigation has been greatly hampered by the lack of a reasonably accurate method of quantitative estimation, without which no real advance could be made. From this difficulty the plant chemists found a partial way out by determining the relative amounts of inorganic and organic phosphorus, though this could only give the most indirect knowledge of the metabolic significance of inositol. However, as far as the animal body was concerned, in spite of the lack of an estimation-method, Starkenstein [1909] concluded that inositol "has no special physiological significance in metabolism." Rosenberger [1910], from an experimental basis even more doubtful, put forward the contrary view that inositol subserved some most important function.

In view of such facts as these, it seemed desirable to continue the search for a method, as the really essential preliminary to an extended study of the general physiological meaning of the inositols.

ATTEMPTS TO EVOLVE A METHOD ON A NEW PRINCIPLE.

The first observation which seemed to be hopeful was found in the work of Neuberg [1908], who succeeded in obtaining furfural from inositol. Some such procedure as that used in the estimation of pentoses might have been possible, but on looking up the original paper, it was found that the change was anything but quantitative, necessitating dry distillation with phosphoric anhydride, and only yielding very small amounts of furfural. Rosenberger in 1910 had hoped that the hexacetate of inositol prepared by Maquenne in 1887 might be applicable to a quantitative method, but this also seemed useless, because of the tri- and tetra-acetates formed at the same time—at any rate for the minute quantities of inositol found in tissue extracts.

A colorimetric method would have been most desirable so the colour-tests for inositol were examined carefully. The most obvious thing about them is that they all depend on the reactions of the oxidation-products of inositol, rhodizonic acid, and other quinonoid bodies. Scherer's original test [1852], modified successively by Mayer [1907] and Salkoffski [1910], consists essentially of a rapid oxidation with strong boiling nitric acid, followed by the formation of calcium rhodizonate (?) which is brick-red or pink in colour. The reactions of Meillière [1906, 1, 2, 3] and of Gallois [1865], are exactly the same in principle, but the red mercury salt is formed instead; while Perrin's test [1909] depends on the formation of a reddish silver salt. Denigés [1907], after oxidation with nitric acid, adds potassium hydroxide, sodium nitrocyanoide, and acetic acid; the solution is said to change from blue to brown to red; Seidel [1884, 1887] reported good results from the use of strontium acetate. Hugo Müller's procedure is the only one which does not utilise oxidation with nitric acid, for here hydrogen peroxide is used instead in the presence of a ferrous salt in traces [1907, 1912].

The writer's experiences with these tests were far from satisfactory. Müller's reaction could be ruled out at once for the purpose in hand, for the products were known to be many and various, oxalic and other acids being produced as well as quinones. In spite of the utmost care it was impossible to get Denigés' test to work at all, and the same applied to the test introduced by Seidel. Seidel's paper was unfortunately inaccessible to the writer, but he was informed by Dr Rosenheim that in the original description of the test, strontium acetate does not appear, and the text-books have described it wrongly. It is surprising that the "Analyse des Harns" of Neubauer and Vogel, usually so accurate, should have fallen into this mistake. What Seidel really used was sodium acetate and barium chloride, in which case the reaction is simply a variant of the Scherer test and is easy to perform.

The Scherer reaction itself, the oldest and most used of all, seemed at first likely to be of some use quantitatively. It appears that a chloride (Ba, Ca, Sr, Al) is necessary, as well as traces of a catalyst such as platinum or a mild oxidising agent like chloramine-T. With the aid of these sugges-

tions and of Mayer's modification, the comparative delicacy of the test was investigated, and it was found that only down to 1 in 3000 were the results uniform and constant. Below that dilution, unknown factors seemed to possess great influence. Furthermore, Mayer [1907] reported failure of the test with a sample of pure inositol, and it was found in this work that quite small quantities of lead salts completely inhibited the reaction. The reaction could not be applied, then, to the estimation of inositol in tissue extracts, or in any even moderately complex mixture.

In consequence, it was necessary to fall back on the classical method for the isolation of inositol; precipitation with basic lead acetate and subsequent decomposition with hydrogen sulphide. This has been used in the past by many workers, including Marmé [1865], who introduced it; Gallois [1864], Külz [1876], Rosenberger [1908, 1, 2] and Starkenstein [1909]. As the basis for a method of estimation it suffered from the fact that it had never been shown to be quantitative, though Meillière [1906, 2] assumed that it was, and Starkenstein [1909] referred to it as "fast quantitativ." Meillière [1907] had reported that the copper acetate compound of inositol studied by Thudichum was insoluble, and could be quantitatively precipitated: the writer's experiences with this are recorded below. In such attempts at quantitative work as had been done, the lead precipitation had been used in the course of "estimation by weighing the isolated product." This of course is an inaccurate procedure, and requires the taking of large amounts of initial material, thus effectively masking any small variations in the tissue; to say nothing of the time consumed in working up the resulting extracts. It therefore occurred to the writer that if the lead precipitation were united to a good method of extraction, and to a micro-carbon estimation at the final stage, some progress might be made with the general problem. The following experimental work was accordingly carried out.

THE PROCESS OF EXTRACTION.

All the earlier workers were accustomed to use extractions of hot and cold water. W. Müller [1857] departed from this practice, however, by grinding wet brain tissue with neutral lead acetate, and Rosenberger [1908, 2] by boiling the tissue with potash, and subsequent alternate treatment with nitric acid and baryta. Both methods were most unsatisfactory; the former because it gave very impure samples of inositol, and the latter because of its drastic nature—"any inositol, which has survived this prolonged treatment," as Momose [1916] says, "being precipitated by lead in the usual way." Momose, working on a suggestion of Rosenheim's, discovered that very good yields of inositol from brain tissue were to be obtained by working up the first watery acetone extract. By this means, inorganic salts, known to interfere with the lead precipitation, were much reduced in quantity, and, as very little else besides inositol was extracted, the crude product was purer.

To find whether this procedure was, or could be made, quantitative, 6 kilos. of butcher's beef muscle was carefully freed from fat, finely minced, and extracted with acetone in the proportion of 1.25 litres per kilo. This, of course, amounts to extraction with dilute acetone, since the muscle contains about 70 % water. After standing all night the strained and filtered liquid was evaporated under diminished pressure to remove all the acetone, and the aqueous residue was worked up for inositol in the usual manner, *i.e.* precipitation successively with neutral, then with basic, lead acetate; decomposition with hydrogen sulphide, and finally precipitation with alcohol and ether. During the stages of the process, qualitative tests were made to show the presence and behaviour of other possible extractives; these are given in Table I.

Table I.

	After removal of acetone	After precipitation with neutral lead acetate	After precipitation with basic lead acetate	After addition of alcohol (absolute)
Reducing sugar	-	-	-	-
Inositol	+	+	+	+
Creatinine	++	++	+	-
Creatine	+	+	-	-
Glutathione	++	-	-	-
Glycogen	-	-	-	-
Lactic acid	-	-	-	-
Cholesterol	-	-	-	-
Purine bases	-	-	-	-

Meanwhile the tissue was again extracted by shaking with acetone in the same proportions for about four hours and allowing it to stand thus overnight. The second acetone extract was treated in exactly the same manner as the first, except that 2500 cc. of water was added to compensate for the absence of the tissue water in the first instance. Then the tissue was extracted a third time, but with water. The results appear in Table II.

Table II.

		Amount of inositol isolated in g.
Extract 1.	Acetone and water	0.935
" 2.	" " "	nil
" 3.	Water only "	nil

It was afterwards found that tissue should not be allowed to remain in the acetone longer than three days; for cholesterol begins then to be extracted in spite of the water present.

It seems justifiable, then, to conclude from these data, that the process of extraction with dilute acetone succeeds in removing from the tissue all the inositol which it contains. The sample obtained in the experiment given above melted at 221° and when examined in the polarimeter, in comparison with a sample of *i*-inositol prepared from phytin, failed to exhibit any optical activity. The conclusion is that the inositols of ox muscle, ox brain, human brain, and phytin, are identical, the last three having been shown to be so by Momose [1916].

THE PROCESS OF PRECIPITATION.

To prepare a supply of inositol, which could be drawn upon for blank experiments, a quantity of commercial phytin was procured and hydrolysed under pressure, bearing in mind the experiences of Plimmer and Page [1913] with organic phosphorus esters. Into hard glass tubes were placed 50 g. of phytin, with 200 cc. of 35 % sulphuric acid; these were sealed and heated for nine hours at 170°. Afterwards, the magnesium and calcium sulphates were filtered from the reaction mixture, the sulphuric acid removed as barium sulphate, excess of baryta as barium carbonate, and the inositol brought down by the addition of alcohol. From 50 g. of phytin, 8.4 g. of inositol were obtained; or about 84 % of the theoretical yield, assuming that Anderson's formula for phytin is correct [1912]. This inositol (M.P. 219°) was used for the following series of experiments. Since, as has been noted above, traces of lead interfere with the Scherer reaction, it was not possible to depend on it as an indication of the presence of inositol, and in each case it was necessary to evaporate the solutions to low bulk and precipitate the inositol, if it was present, with absolute alcohol or ether.

In order to approximate as much as might be to the actual conditions of tissue extracts, the concentration was made about the same as it would be expected to be from 200 g. of muscle. 8 cc. of a standard 0.5 % inositol solution were diluted to 140 cc. with distilled water, thus allowing for the water of the tissues. To this the saturated basic lead acetate solution was added, the conditions being varied. A preliminary test was done to see if the addition of 500 cc. of acetone, and its removal, had any effect on the inositol content. *A priori*, this was most unlikely, as inositol is insoluble in acetone; and colour-tests showed that it was not the case.

Table III.

	Inositol found in the filtrate after decomposition of lead compound with H ₂ S and precipitation by alcohol
Basic lead acetate, saturated solution boiled	
equal quantity, or less	++ +
excess	++
excess + concentrated ammonia 3 cc.	++
excess, reprecipitated 3 times	++
excess left to stand three days	+++
Basic lead acetate, saturated solution unboiled	
equal quantity, or less	+ -
excess	+ -
excess + concentrated ammonia 3 cc.	-
excess + reprecipitated three times	-
excess left to stand three days	++

Inositol tends to be held, perhaps in an adsorbed state, by the precipitate of lead sulphide, requiring two extractions with water at about 70° to remove it completely. It is readily adsorbed by Fuller's earth, though not by kieselguhr. Several points are clear from the above results. In the first place, Momose's observation that the lead acetate compound dissociates on prolonged washing receives corroboration from the fact that the precipitation

seems to fail when the mixture is not filtered for three days. The ordinary hydrolysis of the basic salt to the hydroxide is presumably much intensified by boiling, hence the importance of using a cold unboiled saturated solution. Meillière showed that the presence of ammonia was important in the precipitation, and his observation was fully confirmed. The quantities of inositol appearing on the addition of absolute alcohol were too small to be weighed; and when no trace of cloudiness appeared, the result was recorded as negative.

To effect a further separation the well-known insolubility of inositol in alcohol was made use of. By evaporating to low bulk (less than 20 to 30 cc. if quantities of the order of 40 mg. as here, are present) and the addition of 300 cc. of absolute alcohol, the inositol present is all precipitated, and the resulting slight cloudiness flocculates well after half-an-hour's standing. In this connection the statement of Cooper-Lane [1861] could not be confirmed. He considered that if too much alcohol was added, the inositol failed to come out of solution; but this was never found to be the case if the watery solution was taken down to a sufficiently low bulk. Reprecipitation after adding a little water to the filtered liquid, and distilling off the alcohol, never gave any further cloudiness.

After standing overnight to ensure complete precipitation of the inositol, it is filtered off from the alcohol by passing it through a carefully prepared Gooch crucible, with very small holes, containing a layer of the cleanest asbestos about three millimetres thick. The air current drawn through by the filter-pump is allowed to pass for 10 minutes to make sure that the asbestos and inositol are quite free from alcohol; and the content of the crucible is then quantitatively transferred to a 500 cc. flask in which it is shaken with about 60 cc. of distilled water. At the end of 10 minutes the inositol is completely dissolved and the asbestos is then filtered off. The solution contains all the inositol from the tissue taken—and nothing else—and, after being made up accurately to 100 cc. is ready for estimation by the micro-carbon part of the method. Numerous tests, carried out on the various stages of this part of the whole method, showed that the inositol could be transferred thus from alcohol to water, without the least loss.

The lead precipitation described above proved satisfactory and could always be relied on. Thudichum [1864], considered precipitation by copper acetate to be quantitative if the inositol solution was evaporated to low bulk on a water-bath with about twice its volume of saturated copper acetate solution, until no more of the green flocculent precipitate was formed. The degree of accuracy which this method possesses can be gauged from the following typical example:

Calculated amount of inositol in the solution per cc. = 25 mg.

Found by the copper precipitation = 24.2 mg.

The method gives low results when checked against the lead precipitation, and is more limited in its application than the latter, since it fails in the presence of traces of sugar.

THE PROCESS OF MICROESTIMATION OF CARBON.

Faced with the problem of estimating these small quantities of inositol in pure solution, it occurred to the writer that the quickest way of proceeding would be to ascertain the carbon-content by means of a micro-combustion method, and to calculate the inositol content from that. Evaporation and subsequent weighing in a platinum dish would of course have solved the difficulty, but the time required in drying to constant weight seemed to contraindicate this method. The method used [see Needham, 1923], cannot be termed a wet combustion in the strict sense, for that would imply one of the chromic acid oxidation methods; what was done was to evaporate a known amount of the solution in the actual boat of the combustion furnace.

The micro-methods collected and described by Pregl [1917] and others, were here of little use, for they all apply to combustions of the dry material, and to deal quantitatively with such small amounts of it as 20 mg. or less, would be very difficult. An expensive microchemical balance would be necessary for the preliminary weighing out, and the final measurement, being gravimetric, would estimate such small quantities of carbon dioxide less well than a burette measurement. These difficulties were overcome by the aid of a method of combustion from solution, and the measurement of the gas evolved, by volume.

In order to check the accuracy of the whole process, from beginning to end, it was necessary to carry out all the operations on a solution of known strength. For this purpose 8 cc. of a 0.4 % solution of inositol were taken, diluted to 140 cc. and the whole method proceeded with as described above. The calculated concentration of inositol in the final pure aqueous solution amounted to 32.00 mg. %. From 1 cc. of the liquid 0.231 cc. of CO_2 were given off on combustion. This, when translated into terms of concentration of inositol, is equivalent to 31.70 mg. %.

THE METHOD APPLIED TO SOME TISSUES OF THE RABBIT.

Two rabbits, *A* and *B*, *B* freshly killed, and *A* dead two days before, were taken and estimations performed as described: on thigh-muscles, heart, kidney and liver. The following figures were obtained:

Tissue	Rabbit	Cc. of CO_2 finally evolved	Tissue originally taken in g.	mg. %		g. per kilo
				Of final solution	Of original tissue	
Muscle	<i>A</i>	0.241	100.0	16.1	16.1	0.16
"	<i>B</i>	0.171	121.0	11.5	9.3	0.10
Heart	<i>A</i> and <i>B</i>	0.234	15.5	10.9	100.6	1.00
Liver	<i>B</i>	0.209	85.5	15.6	16.3	0.16
Kidney	<i>A</i> and <i>B</i>	0.163	35.5	14.0	30.6	0.30

Until more extended investigations have been made, it is impossible to appraise the exact significance of the above figures. For example, the individual variations of normal rabbits are as yet unknown. At the same time, while bearing this in mind, it is certainly interesting that the muscle from the

freshly-killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger [1908, 1, 1910], that tissues contain an "inositogen"; though his conclusions were drawn from the comparison of fresh rabbit muscle, and commercial beef; tissues perhaps hardly comparable. Unfortunately, there are no previous figures with which to compare the above. But it is worth remembering that Marmé [1864], reported specially good yields of inositol from heart muscle.

Obviously, the applicability of the method as a whole, may not be general. In extracts of plant tissues, for example, or of animal tissues, not yet investigated, there might well be substances present, which, extracted by dilute acetone, and carried down by basic lead acetate, would seriously affect the final result. But as far as the data go at the time of writing, the general principle seems safe.

SUMMARY.

1. A method is described for the estimation of *i*-inositol in animal tissues.
2. Preliminary results, obtained by the aid of the method on some of the tissues of the rabbit, are given.
3. The inositol of ox muscle is identical with that which is produced by the hydrolysis of phytin.

The writer wishes to take this opportunity of thanking Professor Hopkins for his interest in the work. He acknowledges also with gratitude much advice received from Mr J. B. S. Haldane on gas analysis, and from Dr O. Rosenheim on the chemical behaviour of inositol. Mr Morven Busby rendered valuable assistance with certain of the experiments. Lastly, the writer's thanks are due to the Clayton Aniline Company, representing the Basel Society of Chemical Industry, who were good enough to supply the phytin used in the work.

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LI. A NOTE ON THE ESTIMATION OF THE CARBON-CONTENT OF SOLUTIONS.

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THE method to be described in this note was evolved to meet a special case, namely, the estimation of very small quantities of a carbon-containing compound in pure solution. In the first of a series of papers on inositol [Needham, 1923], the causes which led to the working out of the method are discussed, and it will be sufficient to say here that it was desired to know the carbon-content of amounts of solution containing as little as 20 mg. or less of *i*-inositol. But the method has also proved applicable to any solution of unknown carbon-content, and has been used successfully by other workers in the laboratory in dealing with bacterial culture media, etc. [Stephenson and Whetham 1923].

The method is not a wet combustion in the usual sense, for that would imply oxidation with chromic or sulphuric acid and weighing the gas evolved, as in the method of Tangl and Kereszty [1911]. Rather it is a combustion from solution, for an accurately measured out quantity of the liquid is evaporated from the receptacle in which the combustion is to take place. A wet combustion in the strict sense would not have been so useful in the case of inositol, because of its great stability towards mineral acids, but in some cases would be no doubt preferable. The sulphuric or chromic acid methods appear to possess no advantage in the matter of time and although the use of a silica tube is avoided, yet a special and somewhat complicated piece of apparatus is necessary for the carrying out of the decomposition.

The central point of the apparatus is a quartz combustion tube filled in the usual manner with rolls of copper gauze and a quantity of copper oxide in wire-form. The boat is of silica and may vary in size; into it is run from an accurate micro-burette a known amount of the solution to be estimated. Throughout the process of combustion, which is over in 20 minutes, a stream of oxygen is passed through the apparatus. A wash-bottle of concentrated sulphuric acid, towers containing soda-lime and sticks of dry potassium hydroxide, and a baryta wash-bottle to indicate the presence of any trace of carbon dioxide come between the oxygen-source and the furnace. On the other side of the furnace the gases first pass through a bulb to collect the condensed water, furnished at the bottom with a tap so that after every estimation the water produced can be run off, and no risk incurred of its

absorbing carbon dioxide in subsequent experiments. True, it could absorb a small amount during the combustion which produced it, but this danger is the less, as, having just been condensed from the state of vapour, it is hot. Next, the gases pass through a bulb containing sulphuric acid, and a U-tube filled with calcium chloride. Between these and a long cooling coil there is placed a screen about five feet square, made of wood, and covered with thick asbestos on both sides. After the cooling coil comes a 2-way tap which can direct the gas either through an index-bubbler or through the absorption apparatus. This, shown in greater detail in the appended figure, is an ordinary

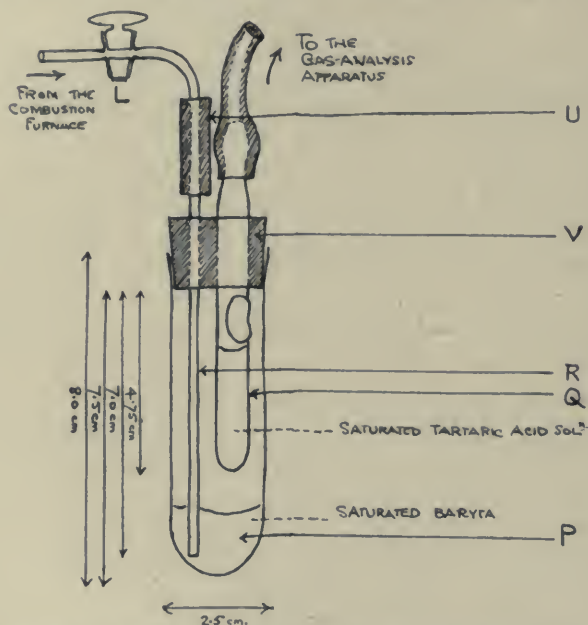


Diagram of the absorption-bulb in the combustion method.

Total internal capacity of bulb =	32.5 cc.
Space occupied by tubes =	4.0 cc.
" " " baryta =	5.0 cc.
" " " acid =	1.0 cc.
	10.0 cc.
	10.0 cc.

Value employed in solubility correction 22.5 cc.

large thin glass test-tube, drawn out and rounded, and is attached to a Haldane gas measuring apparatus, made exactly as described by Haldane [1920]. The tube connecting with the furnace has another tap through which the gases are led on down the tube *R* to the bottom of the absorption bulb, through the rubber cork *V*. *U* is a collar of rubber tubing to permit of a firm hold for the crucible-tongs when the bulb is shaken in the process of evolving the gas. Exactly 5 cc. of saturated baryta are placed in the bottom of the flask as shown in the diagram so that the tube *R* dips down into it. The tube *Q*, containing 1 cc. of a saturated solution of tartaric acid, which connects with the measuring burette, is pierced by an aperture just underneath the

cork V, large enough to permit an easy shaking out of the tartaric acid, and sufficiently small to prevent any escaping before the proper time. The carbon dioxide, then, produced by the combustion of the carbon-containing compound, is dried, cooled, and caught by baryta in such a manner that it can subsequently be driven off and measured. Since it is one of the heavier gases, it tends to collect at the bottom of the cooling coil, but it is readily washed out from there by a rapid stream of oxygen passed through at the conclusion of the combustion.

First, the blank of the whole apparatus was determined. In all cases whether blank or not, the procedure was exactly the same. The results here given are corrected for solubility of CO_2 in the saturated barium tartrate solution, and reduced to N.T.P. Haldane describes the corrections in full.

Blank experiments (empty boat, or boat with distilled water).

Exp. No. IV	12	0.082 cc. of gas evolved
	13	0.070 " "
	14	0.075 " "
	16	0.090 " "
	18	0.102 " "
	19	0.091 " "
	20	0.084 " "
	21	0.095 " "
	22	0.088 " "

Average = 0.081

This blank value was redetermined from time to time, and grew more constant as the apparatus was used. Its high figure cannot be accounted for by traces of CO_2 or by the "reagent blank" (no oxygen stream or heating) for the latter is very constant at 0.015. If oxygen is passed through without heating the combustion tube, but keeping all the other conditions constant, the blank is still at 0.015, so the most probable supposition is that small quantities of carbon monoxide from the oxygen cylinder are burnt in the tube and appear as carbon dioxide. An iodine pentoxide tube would have avoided this, but the constancy of the blank was thought to render it unnecessary.

The next thing that was done was to place in the tube accurately measured out amounts of a solution of known strength of some pure carbon compound and carry out combustions on them. Inositol was chosen.

0.1 cc. of a 0.5 % solution of inositol (equivalent to 0.5 mg. inositol); gas evolved in cc. 0.333, 0.391, 0.408, 0.370, 0.382; average = 0.377; calculated value = 0.374.

0.2 cc. of a 0.5 % solution of inositol (equivalent to 1 mg. inositol); gas evolved in cc. 0.761, 0.718, 0.716; average = 0.730; calculated value = 0.758.

The micro-combustion method was then checked against the evaporation of the final solution, first in a porcelain basin, and then in a platinum dish; followed by weighing the residue before and after incineration. The figures of a typical example are as follows:

From 5 cc. of solution, there were evolved $\left\{ \begin{array}{l} 0.310 \text{ cc. of gas.} \\ 0.326 \quad \quad \quad \text{,,} \end{array} \right.$

The solution therefore contained 9.19 mg. of inositol %.

Weight of platinum dish	24.5300 g.
„ „ after evaporation to dryness	24.6020 g.
„ „ after incineration	24.5928 g.
Weight of organic content	9.2 mg.

This method has been used to obtain preliminary data concerning the inositol content of the tissues of rabbits: the figures are given in the other paper.

As against the process of evaporating to dryness and drying to constant weight, it possesses great advantage in the matter of time. From first to last the method of combustion from solution requires only a little over half an hour, whereas the alternative procedure demands a very much longer wait.

It is to be hoped that the method above described will prove useful in the future to workers desiring to estimate the carbon-content of quantities of liquid containing less than 20 mg. of the carbon-containing substance per 100 cc.

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LII. THE EFFECT OF INSULIN ON THE GLYCOGEN IN THE TISSUES OF NORMAL ANIMALS.

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THE immediate and most striking effect of insulin when injected into normal animals, is the fall of blood sugar.

Experiments made by one of us in collaboration with Laidlaw, Boock and Trevan [1923] suggest that this disappearance of sugar is not due simply to an increased combustion of carbohydrate, since the output of carbon dioxide and the oxygen consumption of normal animals under the influence of insulin both fall continuously and rapidly to a very low level, while the respiratory quotient, after a short, transient rise also falls. Kellaway and Hughes [1923] studying the normal human subject, which behaves in a similar way, consider that the glucose disappears, not because it is burnt, but rather because it is converted into some complex substance.

The recent suggestion of Dale [1923] that the action of insulin is to shift the incidence of combustion on to the carbohydrate and to inhibit or at any rate spare the normal catabolism of fats and proteins must be borne in mind and experiments to test this hypothesis are being performed.

The evidence, as far as it goes, being rather in favour of the view that the vanished glucose has not left the body as carbon dioxide and water, it was anticipated that possibly the effect of insulin might be to favour the laying down of glycogen by the liver, as is apparently the case in diabetic (depancreatised) dogs [Banting and colleagues, 1922], and that consequently the glucose of the blood might be found as glycogen in that organ. But on putting the matter to the test, it was found that not only is the sugar not stored in this manner, but the livers of normal animals which have received sufficient insulin to bring them to the verge of hypoglycaemic convulsions contain very little glycogen. It appears, in fact, that as soon as the blood sugar falls to a certain level the liver mobilises its glycogen and pours glucose into the blood stream, in an effort to maintain an adequate concentration of sugar there.

The possibility of a transference of glycogen from the liver to the skeletal muscles and a temporary storage of glycogen in them was considered and

investigated. Again, as in the case of the liver, a disappearance and not an accumulation of glycogen was observed. The experiments on this question seem to indicate that the glycogen of the muscles remains more or less unaffected until that of the liver is greatly depleted, and that then the muscle glycogen is called upon to provide glucose for the circulating blood.

The heart muscle, as might be expected, appears to retain its glycogen to the last. It may be mentioned that the fat content of the livers of mice which had been given large doses of insulin was compared with that of the livers of normal mice. No difference either in the quantity of fat or its degree of unsaturation, as indicated by iodine values, was detected. Apparently, then, the effect of insulin is not to cause a conversion of carbohydrate into fat. Further experimental work on the fate of the sugar in normal animals under the influence of insulin is being carried out.

EXPERIMENTAL.

The glycogen content of the livers of normal mice and of mice after insulin.

Three normal mice were killed, the livers rapidly removed and worked up separately by Pflüger's method. The sugar in the hydrolysed fluid was estimated by Shaffer and Hartmann's micro-method [1920].

Weight of mice in g.	Weight of livers in g.	% glycogen in liver
24	1.79	2.02
21	1.47	2.36
18	1.22 (cyst in liver)	0.89

Three mice were each given 1/400 mg. of "insulin picrate" [Dudley 1923] subcutaneously, and were killed immediately on the appearance of convulsions, which happened within an hour of the injection.

Weight of mice in g.	Weight of livers in g.	% glycogen in liver
23	1.17	0.075
21	1.15	
20	1.12	

As preliminary qualitative experiments had shown the disappearance of glycogen from the livers of such mice the three livers were combined and worked up together.

It will be seen that the average glycogen content of these livers is less than one-tenth of the lowest of the three control animals. It should be noted that there was a large cyst in the liver of the third control animal; this may account for the glycogen content of its liver being so much below that of the other two controls, whose livers were quite normal.

This experiment has been performed several times and the result is always the same. A quantitative determination of the glycogen is not necessary to convince one of the main fact of the disappearance of glycogen under the

influence of insulin. On precipitation of the glycogen with alcohol it is at once strikingly apparent that the glycogen of the insulin-treated livers is much less than that of the normals.

Glycogen in the liver and muscles of the normal and insulin-treated rabbit.

Two rabbits, which had been well fed on carrots and beetroot for a couple of days previous to the experiment were used in this experiment. One of the rabbits, weighing 2.3 kilo. received an injection of 10 mg. (10 2-kilo. rabbit units) "insulin hydrochloride" [Dudley, 1923]. It developed hypoglycaemic convulsions six hours after the injection. It was then killed. The other rabbit, weighing 2.8 kilo., which had been kept in a cage without food from the time of the injection of the first rabbit, was killed at the same time. The livers, hearts, and about 50 g. of thigh muscle, were removed from the animals promptly and worked up for glycogen according to Pflüger's method.

The following results were obtained:

	Normal rabbit % glycogen	Insulin rabbit % glycogen
Liver	5.53	1.86
Heart	0.26	0.54
Skeletal muscle	0.57	0.0

It will be noted that the glycogen content of the insulin rabbit's liver is much less than that of the normal, and there was so little glycogen in the insulin rabbit's muscle that it could not be estimated.

The glycogen content of the normal rabbit's heart was only about half that of the insulin rabbit's, but no special significance is attached to this result, since it is known that such differences do occur in normal animals [Abderhalden, 1914], and another determination on a normal rabbit's heart gave 0.57 % glycogen.

The fat content of the livers of normal mice and of mice after insulin.

Thirteen normal mice with an average weight of 20.7 g., the extremes being 25 g. and 18 g., were killed by first stunning and then bleeding by cutting the vessels of the throat. The livers, bulked together, weighed 14.91 g. The tissue was hydrolysed in 60 % KOH on a boiling water-bath, a stream of hydrogen being passed through the flask to prevent oxidation of the unsaturated fatty acids. The hydrolysed liquid was strongly acidified with HCl and the fatty acids were shaken out with ether. The ethereal solution was thoroughly washed with water and then evaporated to dryness *in vacuo*, a brownish crystalline mass being obtained. This was dissolved in 50 cc. of absolute alcohol.

Thirteen mice with an average weight of 21.5 g., the extremes being 24 g. and 19 g., were injected subcutaneously with insulin hydrochloride (1/1000 mg. per gram of mouse) and placed in a room warmed to 28°. In three-quarters of an hour most of the mice were dead and the rest moribund. Their livers were

collected and weighed 18.95 g. The fatty acid was extracted by the same technique as in the case of the normal mice livers and the resultant fatty acid was dissolved in 50 cc. of absolute alcohol.

10 cc. of each of the solutions of fatty acid were titrated with alcoholic N/10 KOH using phenolphthalein as indicator and the amount of KOH required was practically identical in each case; namely, 5.4 cc. for the control and 5.32 cc. for the insulin experiment.

The remaining 40 cc. of alcoholic solution were in each case evaporated to dryness and the residues were taken up in chloroform.

Iodine values were determined by Hübl's method. There was no significant difference in the volumes of $\text{Na}_2\text{S}_2\text{O}_3$ required to titrate the excess iodine.

A comparison of the weights of the liver taken in these experiments is not justifiable since the insulin livers, which weighed considerably more than the normal livers, were obviously gorged with blood. The total weight of the normal mice was 269.1 g. and that of the insulin mice was 279.5 g. It is therefore a fair assumption that the total weight of liver tissue taken in the two experiments was the same within, say, 5 %. Therefore, as the amounts of fatty acid obtained were practically identical in both cases, the experiment shows satisfactorily that the sugar which disappears under the action of insulin is not converted into fat in the liver.

We are greatly indebted to Dr H. H. Dale for valuable help and advice during the course of these experiments.

Since this paper was written we have learned that Professor Babkin, working independently at University College, has been studying the same problem, and that his results are in agreement with ours.

SUMMARY.

1. The sugar which disappears from the blood of normal animals under the influence of insulin is neither converted into nor stored as glycogen either in the liver or in the skeletal muscles.

2. The glycogen of both liver and skeletal muscle disappears almost completely in animals which are given a dose of insulin sufficient to cause hypoglycaemic convulsions.

3. There is no evidence of a conversion of carbohydrate into fat in normal animals under the influence of insulin.

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LIII. THE ABSORPTION AND TRANSLOCATION OF LEAD BY PLANTS.

A CONTRIBUTION TO THE APPLICATION OF THE METHOD OF RADIOACTIVE INDICATORS IN THE INVESTIGATION OF THE CHANGE OF SUBSTANCE IN PLANTS.

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THE investigation of the absorption of lead by plants can be carried out quite simply by dipping them into a solution which contains a radioactive isotope of lead, and determining the radioactivity of the ash from various parts of the plant. In addition to its simplicity and the extraordinary rapidity with which the work can be carried out this method possesses the following advantages:—(a) By mixing suitable amounts of ordinary lead with the radioactive lead isotope, one can vary the lead concentration of the solution, as it were, between very wide limits. The assimilation of lead from a $N/1$ solution can be just as readily investigated as that from a solution many million times more dilute. (b) One can follow the change in localisation of the lead taken up by the plant, and thence draw conclusions as to the nature of its combination.

The experiments described in this paper were so carried out, that the plants, which had been cultivated in a culture solution, were washed with distilled water, and then the roots were immersed from 1 to 48 hours in a solution containing a mixture of lead nitrate and thorium *B* nitrate. In most cases *Vicia Faba* (horse-bean) was used. After this period of immersion the individual parts of the plant were first well rinsed with distilled water, and then ignited, and the intensity of the radioactivity of the ash was determined by means of an electroscope. This latter magnitude gives directly the lead content of the ash and thus also that of the corresponding part of the plant, when we know the radioactivity and the lead content of the solution in which the plant has been immersed.

Thorium *B* is a transformation product of thorium emanation, and is obtained in a very simple manner. A piece of platinum foil is charged nega-

tively to a potential of 110 volts, say, and suspended in a vessel containing the preparation (radio-thorium, thorium *X*, etc.) from which the thorium emanation is generated. Under these circumstances the thorium *B* collects on the platinum surface, and can be removed with the aid of a few drops of dilute nitric acid. The normality as regards lead of a solution (thorium *B* is an isotope of lead, *i.e.* a substance showing completely the chemical properties of lead) prepared in this way is about 10^{-12} , and if we wish to increase it we only need add to the solution a known amount of lead nitrate. For example, if we assume that we have prepared in this manner a 10^{-6} *N* solution of lead nitrate, and that after evaporating it to dryness it shows a radioactivity of 10,000 relative units, then each relative radioactive unit would correspond to an amount of $2 \cdot 10^{-5}$ mg. of lead. We must of course take account of the fact that the material of the ash of the parts of the plants absorb part of the rays from the contained thorium *B*, but we can easily eliminate this disturbance by mixing the preparation used for comparison with the same quantity of ash as is contained in the sample the radioactivity of which we desire to know.

The following example shows the procedure during an experiment: *Vicia Faba* that had undergone cultivation in a nutrient solution for a fortnight was introduced, after careful washing, into 500 cc. of a 10^{-5} *N* radioactive solution of lead nitrate, which also contained 1/200 mol. of sodium nitrate. The temperature was 17°. The usual precautions such as screening the roots from light, etc., were also attended to. After 22 hours the plant was removed from the solution, and after careful washing with distilled water, the various parts—root, fruit, stem and leaves—were dried separately, ignited after the addition of a drop of concentrated sulphuric acid, and measured electroscopically¹. The result of the experiment is shown in the following table.

Experiment (a):

Part of plant	Weight of ash in mg.	% of the total lead in the solution contained in the ash	Mg. of lead in the ash	Lead content of the ash in %
Roots	45	13.1	0.11	0.25
Fruit	5	0.10	0.0008	0.016
Stem	46	0.05	0.0004	0.001
Leaves	36	0.013	0.0001	0.0003

The purpose of the following experiments was to investigate the manner in which the assimilation of lead in the case of *Vicia Faba* varies with the lead concentration of the solution. In all of these experiments the volume of the lead solution was 200 cc. and the duration of the experiment was 24 hours.

The following collection of results shows that the individual experiments can be repeated, the agreement being quite satisfactory. Per cent. of the

¹ Before the measurement one must wait about six hours in order to be certain that radioactive equilibrium has been established between thorium *B* and thorium *C*. The reasons for this are outside the scope of this paper.

lead content taken up by the root from a 10^{-6} *N* solution: 61.2, 62.3, 57.4, 59.6, 55.4, 57.8, 47.3, 62.2, 61.7, 62.6, 60.0, 51.2, 68.7, 57.6.

Experiment (b). With 10^{-6} *N* lead solution:

Part of plant ¹	Weight of ash in mg.	% of the total lead in the solution contained in the ash	Mg. of lead in the ash	Lead content of the ash in %
Root	41	60.0	0.02	0.052
Stem	12.6	0.04	0.000013	0.0001
Leaves	5.5	0.004	0.000001	0.00002

Experiment (c). With 10^{-5} *N* lead solution:

Root	43	31.7	0.11	0.26
Stem	18	0.015	0.0004	0.002
Leaves	9.8	0.0012	0.00003	0.0003

Experiment (d). With 10^{-3} *N* lead solution:

Root	39	11.9	3.9	10
Stem	18	0.02	0.007	0.04
Leaves	18	0.002	0.0007	0.004

Experiment (e). With 10^{-1} *N* lead solution:

Roots	26	0.30	9.9	38
Fruit	18	0.11	3.6	20
Stem	11	0.065	2.2	20
Leaves	10	0.035	1.2	12

¹ The fruit was removed when the plant was introduced into the culture solution, since it constitutes a particularly good nutritive medium for troublesome moulds.

From the above experimental data it is seen that, whereas in the case of a 10^{-6} *N* solution more than half of the lead is taken up by the root, the percentage loss when a 10^{-1} *N* solution is used only amounts to 0.3, although the quantities of lead taken up by the root in the latter case are very much greater than in the former case. It is of interest to note that the *percentage* of lead which passes over into the stem and leaves from the concentrated solution of lead is not smaller than that from dilute solutions. This can be interpreted as meaning that with very dilute solutions the root itself is able to bind almost the whole quantity of lead, and thus renders extremely difficult the ascent of lead into the stem and leaves. On the other hand, when a concentrated lead solution is used, an ample sufficiency of unbound lead is available, and this can be carried upwards by the transpiration current. Except in the case of concentrated solutions, the root thus protects, as it were, the remaining parts of the plant, and this marked ability for "binding" lead is probably connected with an explanation of the relatively small toxicity of lead for plants, discussed on p. 444¹ [cf. Strasburger, 1891].

ON THE MODE OF COMBINATION OF LEAD IN THE ROOT.

The question as to whether the assimilated lead enters into an organic molecule, or whether it is retained by the plant in the form of a saline com-

¹ Trees placed in solutions of copper sulphate or picric acid, etc., do not die until the poisonous substance has reached the highest points of the crown.

pound can easily be decided. In the first case, lead atoms which had once been taken up by the root would not be able to interchange places with other lead atoms, whereas in the second case an active kinetic interchange between the lead atoms bound in the plant and those present in the solution would necessarily take place.

In order to make the argument clearer, we shall designate the atoms of lead in molecules such as those of lead tetraphenyl as "red" ones, and those which occur in such a form as lead nitrate as "blue" ones. If we dissolve both compounds in the same solvent and then separate them by crystallisation, we should find only *red* atoms in the lead tetraphenyl and only *blue* ones in the lead nitrate, since the lead atoms in the lead tetraphenyl are available in an undissociable form. If, on the other hand, we dissolve equi-molecular amounts of lead chloride (with *red* lead) and lead nitrate (with *blue* lead), *i.e.* two salts in the same solvent, then after separation the two compounds would be composed half of *red* and half of *blue* lead atoms [Hevesy and Zechmeister, 1920]. The distinction between *red* and *blue* corresponds here to radioactive and to inactive lead.

If the root has taken up active lead and we place it in a solution of inactive lead, then, if the active lead lies stably embedded in organic molecules, no active lead will be able to pass over into the solution, or in other words we shall not be able to displace the active lead with the aid of inactive lead. Now experiment shows that, with the help of a solution which is relatively rich in lead (10^{-2} *N*), we can remove almost quantitatively the lead taken up by the root, whence we must conclude that *the lead in the plant root exists in the form of a dissociable saline compound*, perhaps attached to the cell walls.

For example, if we introduce a *Vicia Faba* (after careful rinsing) which has stood 24 hours in 200 cc. of an active 10^{-6} *N* lead nitrate solution into a much more concentrated 10^{-2} *N* inactive lead nitrate solution of the same volume, we find that 95 % of the active lead taken up by the root passes over into the 10^{-2} *N* solution; *i.e.* the active Pb-atoms are almost completely displaced from their places in the root by inactive atoms, which, of course, preponderate strongly (about 20,000 times), from the statistical viewpoint.

Now a 10^{-2} *N* lead nitrate solution is partially split up hydrolytically, and one might be inclined to ascribe the inverse dissolving action of lead nitrate to its acid content. However, with the aid of a 10^{-3} *N* HNO_3 solution it was possible to remove only 29 % of the lead content of the root, and by the use of distilled water as solvent only 18 % could be removed. The investigation of the assimilation of lead from solutions of different lead content showed that from 10^{-4} *N* HNO_3 64 %, and from 10^{-3} *N* HNO_3 practically the same amount, *viz.* 62 % is taken up by the root, when the normality of the lead ions in the solution is 10^{-6} *N*. From a 10^{-2} *N* HNO_3 solution, a concentration sufficient, in general, to kill the plant, only 26 % is assimilated by the root.

THE DISPLACEMENT OF THE LEAD TAKEN UP BY THE ROOT
BY OTHER IONS.

Since it has been established that we can displace the lead taken up by the root by other lead atoms, it seemed to be of interest to investigate the ability of other ions to displace the assimilated lead. 10^{-2} *N* solutions were used throughout these experiments. The plant containing lead was placed for 24 hours in the solution under consideration and then both the amount of lead remaining in the plant and the amount displaced into the solution were determined. The results of these experiments are shown in the following table:

Solution used	% of the lead initially present in the root which remained after treatment
Lead nitrate (inactive)	5
Cupric nitrate	3
Cadmium nitrate	34
Zinc nitrate	38
Chromium nitrate	43
Barium nitrate	74
Sodium nitrate	76

When the reverse solution took place with the help of a 10^{-3} *N* $\text{Pb}(\text{NO}_3)_2$ solution, 14 % of the originally assimilated lead were still present in the root after 24 hours' treatment.

Only copper is able to displace lead in a similar degree to lead itself; all the other cations investigated show an appreciably smaller displacing power.

The extent of the re-solution of the lead taken up by the stem and leaves was not determined. Experiments which are being undertaken on the assimilation of lead by *algae* will, amongst other things, also serve to shed light on this point.

As is well known, different ions are assimilated to quite different degrees by plants, according to what other ions are present in the culture solution. The toxicity of individual types of ions is also arrested by others. One of the best known cases of this "antagonism" is probably that between CaCl_2 and NaCl . In this case the phenomenon of the suspension of the toxicity of NaCl by CaCl_2 is attributed to the ability of the CaCl_2 to alter the plasma-membrane in such a way that it is less permeable to NaCl [Osterhout, 1912]. Since it has been possible to show that in the case of lead a kinetic displacement of the assimilated ions by other ions occurs, we shall certainly have to reckon with the possibility that the antagonism is in individual cases occasioned by such kinetic effects.

LEAD ASSIMILATION AND TRANSPIRATION CURRENT.

From the fact that more than 50 % of the lead is taken up in 24 hours by the root in very dilute solutions of lead, i.e. a quantity of lead which was present in more than 100 cc. of liquid, we can conclude that it is *not the transpiration current* which transmits the assimilated lead, since the daily

loss of water of *Vicia Faba* under present conditions is less than 1 cc. This independence is also shown in the following experiment. In one case the percentage assimilation of lead by the root was determined in the usual way, and in another after the root had been first separated from the stem under water. The volume of water was 500 cc., the lead concentration was 10^{-6} N, and $p_{\text{H}} = 4$. Duration of experiment = 1 hour.

Root as usual	6.8 and 7.5 %.
Root cut off	6.5 and 7.1 %.

It is seen that the amount of lead taken up was in both cases the same. Moreover, the lack of dependence of the assimilation of salt on the absorption of water by the plant has repeatedly been established [cf. Arrhenius, 1922].

THE TOXICITY OF LEAD.

In connection with the experiments described in the previous section it is of interest to note that, as has been shown by Bonnet [1922], the introduction of plants into 10^{-1} N $\text{Pb}(\text{NO}_3)_2$ solution unfavourably influences the transpiration current. In contrast to more dilute solutions, such an appreciable concentration of lead shows distinct toxic effects on the plant¹. *Vicia Faba* which had stood 24 hours in a 10^{-1} N $\text{Pb}(\text{NO}_3)_2$ solution already showed a slight deviation from the geotropic direction, and the leaves situated closest to the root showed signs of withering.

The toxic action of lead on different plants, such as wheat, radishes, lentils, cabbage, etc., has been investigated quite recently by Bonnet [1922]. Just as in the present case, he introduced the plants into 200 cc. of water after their roots had attained a length of several centimetres. The water contained in solution a definite amount of lead acetate or lead nitrate, and he obtained the following results:

- (1) After the plants had stood in 10^{-1} N solutions of lead salts, lead could readily be detected qualitatively in the root.
- (2) Only traces of lead were found in the stem and in the leaves.
- (3) 10^{-1} N solutions of lead killed, *e.g.* the wheat plant after 20 days, balsam after two days.
- (4) Mg, Ca and K showed no antagonistic action to lead.
- (5) The greater the dilution, the less lead was taken up by the plant.

Our present results confirm those of Bonnet. As regards the first result, we were able, thanks to the sensitiveness of the radioactive method, to detect with ease and quantitatively to determine the presence of lead even in the stem and in the leaves. It is interesting to note that Mg, Ca and K, which do not have an antitoxic action, have only a slight capacity of displacing lead, according to the experiments of the present author. In reference to

¹ Cf. also Lavison [1911] and older experiments of Phillips [1883], Knop [1885], Nolle, Bässler and Will [1884].

point (5), the radioactive methods enable us to carry out a quantitative investigation of the dependence of the assimilation of lead on the concentration of the solution within wide limits, in which all other methods fail. In this manner, it is found that only $1/500$ part of the amount of lead is taken up from a 10^{-6} *N* solution as compared with a 10^{-1} *N* solution. Those experiments of Bonnet should be mentioned, from which we can see the influence of the assimilation of lead on the growth of plants. He finds the following values:

Plant: The bean.

	Length of root in mm.		
	Initially	After 1 week	After 1 month
In water	25	100	1000
In 10^{-3} <i>N</i> $\text{Pb}(\text{NO}_3)_2$	31	31	32

SUMMARY.

(1) The assimilation of lead from lead nitrate solutions by *Vicia Faba* has been investigated. A radioactive isotope of lead was mixed with the lead nitrate, and the amount of lead taken up was determined after ignition from the radioactive intensity of the ash of the various parts. This method makes possible the determination of exceedingly small amounts of assimilated lead.

(2) Whereas 0.3 % of the lead is taken up by the root from 200 cc. of a 10^{-1} *N* lead nitrate solution in the course of 24 hours, 60 % of the lead content of a 10^{-6} *N* solution is taken up in the same time. The leaves show a lead content of only a few hundredths or thousandths of 1 % of the amount of lead present in the solution.

(3) The assimilated (radioactive) lead can be displaced by introduction of the plant containing lead into another lead solution, whereby inactive lead atoms now take the place of the radioactive ones. From this it follows that the lead is not combined with carbon within the plant, but that it exists in the form of a dissociable salt which is soluble with difficulty.

(4) Even after 24 hours, a 10^{-1} *N* solution of a lead salt produces toxic effects on the plant, whilst more dilute solutions do not. Lead belongs to the least poisonous of the heavy metals.

I am indebted primarily to Professor Fr. Weis for so kindly placing at my disposal the facilities of his Institute, furthermore to Dr B. Krause for his help, and to my friend Professor L. Zeehmeister for numerous suggestions.

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LIV. STUDIES ON THE FAT-SOLUBLE GROWTH-PROMOTING FACTOR.

(I) STORAGE.

(II) SYNTHESIS.

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(I) STORAGE OF FAT-SOLUBLE A.

It has been known for some time that some land animals which receive the fat-soluble growth-promoting factor in their diet store a certain amount of it in their glandular organs [McCollum and Davis, 1915, 2; Osborne and Mendel, 1917, 1918; McCollum, Simmonds and Parsons, 1921; Zilva and Drummond, 1922] and a lesser amount in their body fat [Drummond, Golding, Zilva and Coward, 1920] and other tissues. Among the glandular organs the chief store-house for this substance is the liver, but the kidneys and testes also contain a moderate amount.

The study here described is part of an investigation into the mode of action of certain forms of light in the promotion of growth and prevention and cure of experimental rickets in rats. In the course of this investigation it was found necessary to learn whether the liver of rats¹ also contains appreciable amounts of fat-soluble A, whether the quantity varies with that in the diet, and whether any is present in the liver of rats that have stopped growing after being fed on a diet as deficient as possible in that factor.

EXPERIMENTAL.

To test liver for the presence of the fat-soluble growth-promoting factor, the biological method described by Zilva and Miura [1921] was employed.

¹ Since this investigation was completed a communication has appeared on the subject of storage of fat-soluble A in the liver of rats [Coward, Lush and Palmer, 1923]. Their findings are very similar to those reported in Part I of this paper.

Rats were given a purified diet free from detectable amounts of fat-soluble *A*, but otherwise adequate when a sufficient amount of the above factor is present.

The diet was composed as follows:

Inactivated caseinogen	20 g.
Starch	50 g.
Cotton-seed oil (hardened)	15 g.
Salt mixture [McCollum and Davis, No. 185, 1915, 1]	5 g.
Marmite	5 g.
Decitrated lemon juice	5 cc.
Distilled water	50 cc.

When growth had ceased for about three weeks, or, after a shorter period if the animal was beginning to lose weight, the rat was considered prepared for the test and the liver feeding was begun. The liver was administered by hand to make sure that the entire amount was consumed. With few exceptions the animals ate it greedily. Liver feeding was continued for from four to six weeks, but for the sake of uniformity and of proper comparison the growth for the first four weeks only is illustrated in the charts.

*The Effect of Feeding 0.5 g. of Liver from Rats receiving 3 %
Cod-Liver Oil in their Diet.*

Rats G. 122, G. 123, G. 126, G. 162, G. 163 and G. 165 were prepared for the test in the manner mentioned above. Of the six rats, five were actually losing weight when the special feeding was started and three had already developed varying degrees of xerophthalmia. To these six rats there was administered daily 0.5 g. of fresh liver from rats fed for from two to four months on a diet containing 5 % cod-liver oil as the main source of fat-soluble *A*. The effect can be seen in Table I and in Chart 1, in which the growth curves of rats G. 123 and G. 126 are illustrated as representative of the group¹.

During the first four weeks of liver feeding these rats gained on an average 15 g. a week, which is equal to the normal for animals of that age. The three rats (G. 122, G. 123 and G. 126) that had xerophthalmia when liver feeding was begun recovered from it in from two to four weeks. In one rat (G. 122) the cure was remarkable inasmuch as xerophthalmia was very far advanced when liver feeding was started.

In order to see whether an appreciable amount of the fat-soluble *A* contained in the liver fed to the above rats had been stored, 0.5 g. of their liver was administered to rats G. 174 and G. 175, previously prepared for the test. There was an appreciable response, but it was not nearly as great as in the case of the rats illustrated in Chart 1.

¹ In the charts only two typical representatives of all the groups are illustrated. The complete details for every group are given in Table I.

Table I. *Showing the total gain in weight of the test rats on — A diet after four weeks of additional feeding with the various amounts and kinds of liver.*

Rat No.	Type of liver tested	Amount of liver fed, g.	Total gain in weight during first four weeks of liver feeding, g.
G. 122	From rats on a diet containing 3 % cod-liver oil	0.5	59
G. 123	" " "	"	44
G. 126	" " "	"	68
G. 162	" " "	"	60
G. 165	" " "	"	71
G. 163	" " "	"	55
G. 174	From rats G. 122, G. 123, G. 126, G. 162, G. 163 and G. 165	"	22
G. 175	" " "	"	28
G. 159	From rats on a diet containing 3 % cod-liver oil	0.25	58
G. 169	" " "	"	73
G. 171	" " "	"	55
G. 170	" " "	"	45
G. 142	From rats G. 159, G. 169, G. 170 and G. 171	"	51
G. 144	" " "	"	36
G. 482	From rats on a diet containing 10 % butter	0.5	30
G. 160	" " "	"	17
G. 177	" " "	"	20
G. 178	" " "	"	18 (2 weeks)
G. 282	From rats on a diet very deficient in fat-soluble A	"	Lost weight
G. 263 a	" " "	"	"
G. 125	" " "	"	"
G. 264 a	" " "	"	3
G. 278	" " "	"	0
G. 314	" " "	1.0	Lost weight
G. 500	" " "	"	"
G. 498	" " "	"	8
G. 166	" " "	"	32
G. 418	From rats on — A diet irradiated for four weeks after they had definitely ceased gaining weight	0.5	23
G. 523	" " "	"	36
G. 525	" " "	"	22
G. 373	" " "	"	19
G. 520	" " "	"	19
G. 375	" " "	"	13

The Effect of Feeding daily 0.25 g. of Liver from rats whose diet contained 3 % Cod-liver Oil.

After preparation, rats G. 159, G. 169, G. 170 and G. 171 were given daily 0.25 g. of liver from rats whose diet had contained 3 % cod-liver oil. Resumption of growth was prompt, and during the first four weeks the gain in weight averaged 14 g. a week, which is equal to the normal for that age. (See Chart 1 and Table I.)

The amount of fat-soluble A stored by rats G. 159, G. 169, G. 170 and G. 171 was now tested by feeding their liver (0.25 g.) daily, to rats G. 142 and G. 144 which had been prepared for the test in the usual way. For the effect, see Table I. Although in these two rats the growth during the first four weeks of liver feeding was greater than in those which received 0.5 g. of more potent liver, yet this is probably accounted for by the fact that rats G. 142 and G. 144 were older and heavier when they were used for the test, and such animals, according to Zilva and Miura [1921], and others, require less fat-soluble A to induce growth. One rat (G. 142) had mild xerophthalmia at the time liver

feeding was begun, but in ten days this had completely disappeared. When liver was discontinued, growth ceased fairly promptly, which indicates that no great amount of fat-soluble *A* contained in the 0.25 g. of this liver fed daily had been stored. (See Table I.)

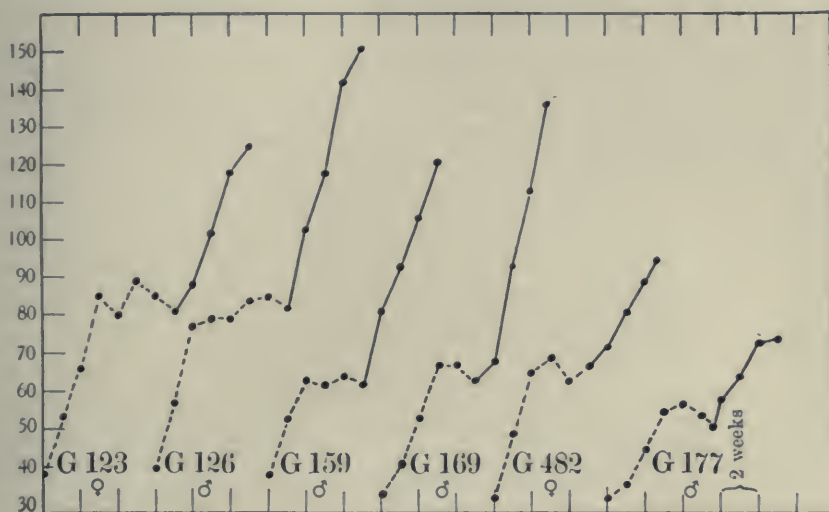


Chart 1.

G. 123 and G. 126. The effect of feeding daily 0.5 g. of the liver of rats whose main source of fat-soluble *A* was 3 % of cod-liver oil in their diet.

G. 159 and G. 169. The effect of feeding daily 0.25 g. of the liver of rats whose main source of fat-soluble *A* was 3 % of cod-liver oil in their diet.

G. 482 and G. 177. The effect of feeding daily 0.5 g. of the liver of rats whose main source of fat-soluble *A* was 10 % of butter in their diet.

Broken line - - - - = Preparatory period on - *A* diet.

Continuous line — = Period of liver feeding.

*The Effect of Feeding Liver from Rats whose Diet contained
10 % of Butter as the chief source of Fat-soluble A.*

Rats G. 160, G. 177, G. 178 and G. 482 were employed for this test and were given daily 0.5 g. of liver from rats fed during a period of from two to four months on a diet containing 10 % butter as the chief source of fat-soluble *A*. The result can be seen in Chart 1 and Table I. The difference between the growth of rats fed on the "butter liver" and that of animals fed on "cod-liver oil liver" is very striking.

Too few rats were available to permit the feeding of their liver to other rats for the detection of storage.

*The Effect of Feeding 0.5 g. and 1.0 g. of Liver from rats that have
ceased growing on a Diet very deficient in Fat-soluble A.*

For this test many rats were placed on the diet deficient in fat-soluble *A* described at the beginning of this communication. When they had definitely ceased growing (no gain in weight for three weeks), 0.5 g. of their liver was

fed to rats G. 282, G. 264 *a*, G. 263 *a*, G. 278 and G. 125 prepared for the test in the usual way. Chart 2 and Table I show definitely that no resumption of growth occurred as a result of this addition to the diet. More rats were tried, but they are not all charted, since some lived for only a week or slightly longer after liver feeding was begun, some developed acute enterocolitis, while others refused to eat the liver after a few days and had to be discarded. None of them showed any resumption of growth; in fact the loss of weight in some was very great on account of the intercurrent intestinal infection.

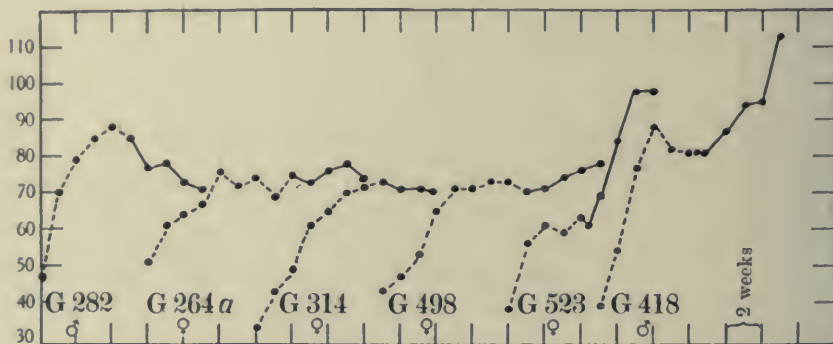


Chart 2.

G. 282 and G. 264 *a*. The effect of feeding daily 0.5 g. of the liver of rats that have ceased growing on the -A diet, very deficient in fat-soluble A.

G. 314 and G. 498. The effect of feeding daily 1.0 g. of the liver of rats that have ceased growing on the -A diet, very deficient in fat-soluble A.

G. 523 and G. 418. The effect of feeding daily 0.5 g. of the liver of rats on the -A diet which were irradiated for four weeks after they had definitely ceased gaining weight.

Broken line - - - - = Preparatory period on -A diet.

Continuous line — = Period of liver feeding.

One gram of similar liver was fed daily to rats G. 314, G. 501, G. 498 and G. 166. Rat G. 314 died after three weeks of liver feeding, having lost slightly in weight during the period. In the case of rat G. 501 liver feeding had to be discontinued after three weeks on account of shortage of material. During the period of liver feeding it did not gain weight. This rat remained on the -A diet and died one week after liver feeding was discontinued. Rat G. 498 gained 8 g. during a period of four weeks of liver feeding, while rat G. 166, receiving the same amount of similar liver, made a definite gain in weight during the first two weeks. After that growth ceased completely, the weight remained stationary, and during the last week the rat developed mild xerophthalmia. The explanation of this gain in weight during the first two weeks may lie in the fact that this rat's store of fat-soluble A was not completely exhausted and that the cessation of growth before the test was begun was not due to deficiency of fat-soluble A alone but to some complication; or, it is also possible that the liver used during that period was from rats which were on the deficient diet too short a time. However, since the deficient diet,

though very poor in fat-soluble *A* cannot be regarded as one containing no trace of that factor, it is hardly likely that the liver of animals fed on such a diet contains none of it. Therefore, it is possible that a rat stops gaining weight before the store of fat-soluble *A* in its body is completely exhausted.

SUMMARY AND CONCLUSIONS OF PART I.

The liver of rats on various diets has been tested by the biological method for the storage of fat-soluble *A*.

1. The results show that the fat-soluble growth-promoting factor, if present in the diet of rats, is stored in their liver, and that it is stored in that organ in rough proportion to the amount contained in the diet.

2. In an indirect way, the conclusion of Zilva and Miura that cod-liver oil is much more potent in fat-soluble *A* than butter is confirmed.

3. Half a gram of liver from rats that had stopped growing on a $-A$ diet showed no appreciable traces of fat-soluble *A* when tested by the biological method. The effect of feeding 1 g. of such liver varied. Thus it would seem that a rat ceases growing before the store of fat-soluble *A* in its body is completely exhausted. Whether that means that this final reserve is used for some purpose other than growth cannot be deduced from these experiments.

(II) ON THE SYNTHESIS OF FAT-SOLUBLE *A* IN THE ANIMAL BODY.

It has been shown by Hume [1922] and the authors [1922] that rats on a diet very deficient in fat-soluble *A* if irradiated for about ten to twenty minutes daily by the mercury-vapour quartz lamp, grow very much better than non-irradiated rats on the same diet. Also, rats on a $-A$ diet, if irradiated daily after they have ceased gaining weight, resume growth for a variable period. But in both cases gain in weight eventually ceases and the subsequent history is the same as that of non-irradiated rats, viz. decline in weight, development of xerophthalmia in some of the animals, and finally death with or without a complicating terminal infection. Because irradiation was unable to effect the continuance of growth on a $-A$ diet, the assumption of a photosynthesis of fat-soluble *A* in the animal body to account for the stimulation of growth seemed unjustified. But it was suggested that the mode of action of light in stimulating growth was "by effecting an economy of the action of the fat-soluble factor still stored in the animal at the time irradiation was begun, as well as by making available the small quantities of organic factor which may still be present in the daily ration of deficient diet."

At the same time that the above investigation was being carried out another method was used to determine whether synthesis of fat-soluble *A* occurs in the body of a rat under the influence of rays from a mercury-vapour quartz lamp.

It has been shown in the first part of this communication that 0.5 g. of the liver of rats on a $-A$ diet that have ceased gaining weight for about three weeks contains no fat-soluble *A* detectable by the biological method, and that

1 g. of such liver gave a positive result in only one out of four tests. Rats were therefore placed on the $-A$ diet, and when they had ceased gaining weight for three weeks, at which time but little fat-soluble A should have been left in the liver, irradiations by the mercury-vapour quartz lamp were begun. In most instances (as reported before) resumption of growth occurred promptly and lasted for a variable period, but rarely for longer than four weeks. Therefore, after four weeks of irradiation, when they had again stopped gaining weight, the rats were killed and their liver was tested in the usual way for fat-soluble A . In the case of rats G. 418, G. 523 and G. 525 the gain was as great or greater than that caused by the same amount of "butter liver" (see Chart 2). Rats G. 373, G. 520 and G. 375 did not show as great a gain (see Table I), but the liver feeding was carried out for only two weeks on account of shortage of material. In all three cases the rats ceased gaining weight quite promptly and died in from three to six weeks after the liver was discontinued. Rats G. 523 and G. 525 were kept on the $-A$ diet for about two weeks after liver feeding was discontinued, during which time they lost weight; and then 0.25 g. of liver from rats receiving cod-liver oil was administered to them. In both cases there was a very prompt gain in weight.

DISCUSSION.

The interpretation of the above results is difficult. For reasons already stated it is not justifiable to postulate actual synthesis of fat-soluble A in the body of these rats¹. If not from synthesis, then where did the vitamin in the liver of the irradiated animals come from? One possibility, which has not yet been tested experimentally, is that it is mobilised from the other parts of the body in which it occurs and is partly stored in the liver. Then why, despite the appreciable store of fat-soluble A still present in their liver, did the irradiated rats not continue to grow, while their liver, when fed to test animals, stimulated the growth of these? Apparently, in the irradiated rats, the vitamin, though still present in the liver cannot be liberated and so be made available to the organism, while that same liver when ingested by another rat becomes a direct source of supply of fat-soluble A immediately available by absorption from the intestinal tract. It must be remembered that during the experimental period of four weeks a test rat consumed about four livers from irradiated animals. But without definite knowledge of how and where in the body this vitamin acts one cannot state why the ingested liver should have this effect. The correct explanation must await the results of further studies on the nature of the fat-soluble growth-promoting factor as well as on the site and mode of its action.

¹ The photosynthetic production, however, of fat-soluble A from some stored material which is not renewed on the deficient diet, is not absolutely excluded.

SUMMARY AND CONCLUSIONS OF PART II.

1. Half a gram of liver from rats on a — *A* diet that have ceased gaining weight for at least three weeks contains no fat-soluble *A* detectable by the biological method.

2. Half a gram of liver from rats on the same — *A* diet irradiated by the mercury-vapour quartz lamp for four weeks after they have definitely ceased gaining weight does contain an appreciable amount of fat-soluble *A* detectable by the biological method.

3. The results of the above investigation are not interpreted as evidence of synthesis of fat-soluble *A* in the animal body.

(Note added on 23rd August, 1923.)

In a preliminary investigation by one of the authors (S.) on the storage of the *antirachitic* organic factor, definite protection against the development of rickets in rats on diet 3143 of McCollum, Simmonds, Shipley and Park [1921] was afforded by the daily administration of 1 g. of liver from rats on purified casein diet which were given 100 milligrams of crude cod-liver oil every day. Partial protection was obtained by 0.5 g. of liver.

But 1 g. of liver from rats, that had ceased growing on the purified diet alone, did not afford any protection against the development of rickets on diet 3143.

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LV. THE TYROSINASE-TYROSINE REACTION.

By HENRY STANLEY RAPER AND ARTHUR WORMALL.

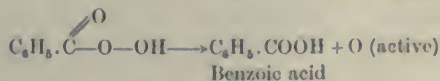
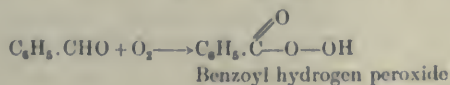
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TYROSINASE, an oxidising enzyme which is widely distributed in the vegetable kingdom and has also been met with in animal organisms, has formed the subject of many investigations since it was discovered by Bourquelot and Bertrand [1896]. The enzyme differs from other oxidation catalysts in that it is able to bring about the oxidation of tyrosine by atmospheric oxygen. Laccase, for instance, which is usually considered to contain an oxygenase-peroxidase system, and hydrogen peroxide and a peroxidase, are without effect on tyrosine solutions. The action of the enzyme on tyrosine is accompanied by the development of a red colour, which gradually becomes reddish brown and finally a black pigment (melanin) is deposited. The nature of the action has been a matter of dispute and has been attributed to a hydrolytic deaminase, a phenolase or oxygenase-peroxidase system, to an enzyme which splits the tyrosine by hydrolysis, and to the inorganic salts associated with the enzyme acting as condensing agents on the oxidation or hydrolytic products produced by the enzyme itself. Into the merits or demerits of these various theories we do not wish to enter at present. Our interest was directed to one important factor concerning the action of the enzyme by the observation that the reaction of the medium in which the enzyme acts has a very marked influence on the colour development both as regards the rate of its production and the tint obtained, and also, that if a partially oxidised tyrosine solution is heated, the red substance first formed is converted into one which is colourless. This colourless substance, while being stable for many hours in an acid solution, rapidly gives rise to a bluish-black pigment on being made alkaline with sodium carbonate. From these simple observations, it was inferred that any investigation of the action of tyrosinase must be carried out with careful regard to the p_{H} of the medium. This is especially true when work bearing on the activity as co-enzymes or inhibitors of various substances or extracts is concerned, and also when the action of tyrosinase on various substances other than tyrosine is being investigated. Our observations have led us to conclude that because of the neglect to work with buffered solutions, a large number of erroneous conclusions have been drawn regarding the enzyme and its action.

The work described in the present paper has defined the limits of p_{H} at which the enzyme is active, and the velocity of the action at three different values of p_{H} has been measured. It has also been shown that the accelerating effect of boiled potato juice on the enzyme action which has been ascribed by Haehn [1919] to the salts present, is not due to the salts but to some organic constituent of the juice, since the ash of the boiled juice does not show the same effect. The results arrived at by Haehn are undoubtedly due to working with unbuffered solutions, a conclusion arrived at independently by Chodat and Wyss [1922].

From what has been mentioned above, it is apparent that previous measurements of the velocity, in which the p_{H} was not kept constant or in which the measurements of the rate of oxidation have been carried out by colorimetric methods, are unreliable. The studies by von Fürth and Jerusalem [1907] and by Bach [1908] come into this category. Bach did not use a colour comparison for estimating the degree of oxidation but employed an empirical procedure by oxidising with permanganate in acid solution the pigment formed during the action of the enzyme. Whilst it is true that the pigment formation from tyrosine is a notable feature of the enzyme action, it is certainly true that more than one pigment is formed, and it is also possible that non-pigmented substances may first be formed from the tyrosine [Bach, 1914]. The permanganate method therefore appears to us to be too crude. The method used in the present work was to control the p_{H} rigidly by using well-buffered solutions, and to estimate the tyrosine at the various stages of the reaction by bromination. In this way, satisfactory curves have been obtained which indicate that as regards the disappearance of the tyrosine, the reaction is of the unimolecular type and that the enzyme is therefore a true catalyst. This observation is of special interest if an oxygenase is a constituent of the enzyme. The oxygenases are supposed to be autoxidisable substances which on exposure to air form peroxides. In the presence of a peroxidase these peroxides are decomposed and yield "active" oxygen. Substances such as aldehydes or unsaturated fatty acids are usually referred to as bodies which are similar to the oxygenases since they are known to form peroxides on exposure to air. They would not function as true catalysts, however, because the peroxides which they form, do not yield the original aldehyde or unsaturated acid on decomposition, but rather, oxidation products of these substances. Thus benzaldehyde gives rise to benzoic acid and active oxygen.



If the oxygenases were of this type, their concentration in the enzyme system would gradually diminish as the reaction proceeded and the velocity

constant would diminish considerably. This we have not found to be the case with tyrosinase and it indicates that if an oxygenase is a component of the enzyme, it acts as a true catalyst and is not permanently altered in bringing about the transfer of atmospheric oxygen either to the tyrosine or to its decomposition products produced by some other component of the enzyme.

Some of the observations recorded in the present paper point clearly to the existence of definite stages in the colour changes which occur when tyrosine is oxidised by the enzyme. These stages appear to be due to the formation of definite compounds which are intermediate between tyrosine and the final product, melanin. They are produced under definite conditions which have now been ascertained and it is hoped eventually to succeed in their isolation and identification. If the enzyme is allowed to act at p_{H} 6, the initial product is bright red in colour and with this reaction of the medium and in the cold, it is fairly stable, changing only slowly into the black pigment which is the end product of the reaction. If the oxidation is carried out at p_{H} 8.0, the red substance does not accumulate, but is rapidly transformed into the black melanin. The red substance, therefore, is to be regarded as the initial product of the reaction. It is not transformed directly into melanin, however, but rather into a colourless substance, which then undergoes oxidation to give melanin. This takes place rapidly in alkaline solutions. The evidence for this is that solutions of the red substance become colourless if allowed to stand in an oxygen-free atmosphere. The change takes place slowly in the cold, more rapidly on warming, and is almost instantaneous when the solution is boiled. If the colourless substance thus obtained be made alkaline, it rapidly darkens in the air, and finally deposits a black precipitate. Bach [1914] has also observed that the red substance is decolorised on heating or on standing in an inert atmosphere, but his conclusions about the change are very different from those we have reached. He attributes the decolorisation of the red substance to a process of reduction and suggests that the reducing agent concerned is a hydrolytic aminoacidase which he regards as a component of the enzyme. Although there appears to be satisfactory evidence that tyrosinase will deaminate amino-acids, the observations of Bach are the only ones which suggest that the action is a hydrolytic oxidation. The evidence submitted by him is, however, unconvincing, and in the present paper experiments are described which show that Bach's conclusions regarding the nature of the enzyme are erroneous. We have been unable to obtain any evidence that a hydrolytic oxidation is taking place. The red substance, it is true, becomes colourless on reduction by SO_2 as well as on simple standing in an inert atmosphere. It could therefore act as a hydrogen acceptor and be reduced if a hydrolytic oxidation catalyst were present. The only evidence submitted by Bach which is in favour of a reduction process is that the decolorisation of the red substance takes place in an inert atmosphere and that if the solution be then shaken in air, the colourless substance is reoxidised and becomes red. To show this, Bach takes a solution of tyrosine, adds tyrosinase and shakes

well for a few minutes at 35–40°. The tyrosine is oxidised and becomes red. The solution is then placed in an atmosphere of nitrogen and becomes colourless (according to Bach, by reduction). On readmitting air and shaking, it again becomes red. According to Bach, this is due to reoxidation of the colourless substance. It may, however, equally well be due to the further action of tyrosinase on tyrosine (both being present), which reaction can now proceed, oxygen having been admitted. That the latter is the true explanation is shown in the present paper. Solutions containing the red substance free from tyrosinase have been prepared. They become colourless on standing in an inert atmosphere, and do not give the red substance again on exposure to air; instead, melanin is produced; slowly if the solution be acid, quickly if it be alkaline. In this experiment, no enzyme is present when the red substance changes to the colourless compound, and the change, therefore, cannot be due to an enzymic reduction. Furthermore, tyrosinase in the presence of tyrosine will not reduce methylene blue in an inert atmosphere. If a hydrolytic oxidation catalyst were present, then the methylene blue ought to act as a hydrogen acceptor and be reduced. The most probable explanation of the above-mentioned colour changes is that the red substance is converted into the colourless compound by intramolecular change, this being slow in the cold, but rapid on heating.

In the light of the evidence presented in this paper, therefore, the action of tyrosinase on tyrosine is divisible into three stages.

1. The conversion of tyrosine into a red substance. This requires the presence of oxygen and the enzyme.
2. The conversion of the red substance into a colourless substance. This is spontaneous, and takes place rapidly on warming. It does not require the presence of tyrosinase and is probably an intramolecular change which the red substance undergoes.
3. The oxidation of the colourless substance by oxygen to melanin. This may take place spontaneously but may also be accelerated by the phenolase which is present in tyrosinase. It is rapid in alkaline solutions, but slow in acid solution.

Experimental Part.

THE SOURCE OF TYROSINASE AND ITS PARTIAL PURIFICATION.

In certain reactions, it is essential that the tyrosinase should be free from some of the substances usually associated with the enzyme, and different methods of purification have been used according to the nature of the substances which were to be removed. In many cases, however, ordinary potato juice was found to give results identical with those obtained when the purified enzyme was used, and in these cases, untreated potato juice was used. This juice was obtained by washing ordinary market potatoes, mincing and then pressing in a meat press. The juice was allowed to stand for a short time and then centrifuged to remove starch.

The purified enzyme solutions most used in these experiments have been obtained by the dialysis of ordinary potato juice. The latter was contained in a very permeable collodion sac suspended in flowing water, and dialysed for four or five days under a slight pressure, this preventing undue dilution of the enzyme solution. The solution obtained in this manner does not give a reaction with tyrosine as good as that given by the original potato juice, but its activity can be increased by vigorous shaking after addition of sufficient sodium carbonate to make the solution 0.1 %.

Another method of purification depended on the digestion of the proteins of potato juice by pancreatic extracts. After four days' digestion in an incubator, the solution was acidified with dilute acetic acid until a definite and approximately maximum precipitate was obtained, care being taken to avoid excess of acetic acid. The precipitate was filtered off, washed with water, and suspended in 0.1 % sodium carbonate solution, in which it dispersed fairly well. It gave a good reaction with tyrosine, though only about half as intense as that given by a corresponding volume of the original potato juice.

Use has also been made of a fairly pure enzyme obtained by repeated precipitation with alcohol to 40 %, and suspension of the precipitate in 0.05 % or 0.1 % sodium carbonate solution. This method probably yields an enzyme preparation similar to that obtained by the method described by Chodat [1912].

THE INFLUENCE OF THE HYDRION CONCENTRATION ON THE ACTION OF THE ENZYME.

It has been found that the hydrogen ion concentration of the medium has a marked influence not only on the velocity of the enzyme action, but also on the nature of the pigments produced from tyrosine.

Buffered solutions containing 0.05 % of tyrosine were obtained from phthalate solutions, phosphate solutions, or boric acid and NaOH mixtures, according to the tables given in Cole's practical *Physiological Chemistry* and Clarke's *Determination of Hydrogen Ions*. 50 cc. of each solution were measured into a 200 cc. Erlenmeyer flask, 5 cc. of fresh potato juice added, and allowed to stand at the room temperature (15°). The solutions were shaken every half-hour, and at intervals were poured out into boiling tubes, any colour changes being noted.

p_H 4.0. No apparent oxidation, *i.e.* no colour changes.

p_H 5.0. Very little oxidation. Slight red colour after 5 or 6 hours.

p_H 6.0. Rapid oxidation. A deep red colour after one hour becoming very intense after 3 or 4 hours. At the end of 24 hours, the solution had become much darker, though the red phase was still very evident.

p_H 7.0. Rapid oxidation. A deep red colour after 1 hour, slightly blacker than that at p_H 6.0. The solution remained blacker than that at p_H 6.0 throughout the experiment and melanin formation was more rapid.

p_{H} 8.0. Rapid oxidation. After one hour, the solution had a red colour, which gradually became darker and more intense. The subsequent blackening and the formation of melanin were more rapid than in the solution at p_{H} 7.0.

p_{H} 9.0. Slight blackening with a little red colour after 2 hours. After 12 hours the solution was very dark, though much lighter than that at p_{H} 8.0.

p_{H} 9.5. Similar results to those at p_{H} 9.0, with corresponding colours of less intensity. Thus there is less visible oxidation than at p_{H} 9.0.

p_{H} 10.0. Very little oxidation. Slight darkening after 6 hours.

p_{H} 10.5, 11.0, 12.0. No apparent oxidation.

The solutions were also tested at intervals for the relative amounts of red substance present in each. This was carried out by withdrawing 5 cc. from each solution, adding one drop of 10 % acetic acid, boiling, filtering and adding sodium carbonate to the filtrate until it was definitely alkaline. By this treatment, the red chromogen is converted into the colourless substance by the process of boiling, which also destroys and coagulates the enzyme, while the addition of the alkali causes the rapid conversion of the colourless substance into melanin. This last reaction yields a bluish-black solution, and the degree of intensity can be regarded as an approximate measure of the amount of red substance present in the original sample.

The results show that the formation of this red chromogen is at a maximum at p_{H} 6.0 to 6.5, is less at p_{H} 7.0, and that the amount then decreases rapidly with increasing alkalinity. As the enzyme action takes place at p_{H} 8.0 with a velocity equal to, or slightly greater than, that at p_{H} 6.0, the small amount of red substance found at p_{H} 8.0 must be due to the rapid conversion of this substance into melanin.

The limits of oxidation of tyrosine by tyrosinase under the conditions given above, are p_{H} 5.0 and p_{H} 10.0 and the enzyme seems to be most active in neutral, slightly alkaline or slightly acid solutions, *i.e.* in the range p_{H} 6.0 to 8.0. Even over this range, however, the colour changes vary enormously.

Chodat and Wyss [1922] working with unbuffered solutions have obtained similar limits of p_{H} for the tyrosinase-tyrosine reaction, though their limit for the alkaline solutions (p_{H} 11.0) is somewhat higher than that observed in the experiments described above.

It is of interest that Mrs E. C. Venn [1920] working with a cheese organism which produced colour changes in tyrosine solutions found that the limits of p_{H} within which the colour was obtained were 3.23 and 9.7. The closeness of these limits to those observed by us suggests that the organism in question produced tyrosinase. The non-correspondence in the more acid media is possibly to be explained by the fact that the enzyme is precipitated in solutions at p_{H} 5.0, whereas it might still be active inside the bacteria.

THE NATURE OF THE CHANGES OCCURRING IN THE
TYROSINASE-TYROSINE REACTION.

The first process in the oxidation of tyrosine by tyrosinase is the formation of a red pigment and this only takes place in the presence of tyrosinase and oxygen. The formation of this red substance can best be shown by the partial oxidation of a saturated tyrosine solution at p_{H} 6.0 by means of one-tenth its volume of fresh potato juice. This product is relatively stable in acid solutions, but in an alkaline medium it is rapidly converted into melanin, probably with intermediate formation of a colourless substance.

If a partially oxidised tyrosine solution at p_{H} 6.0 is treated with colloidal ferric hydroxide to remove the enzyme and other colloidal substances, a clear deep red solution is obtained which is fairly stable, but on standing several hours it deposits a black pigment, melanin. This blackening, which is a process of oxidation, continues until all the red colour disappears. At higher temperatures such as 35–40°, the decolorisation of the red substance is more rapid, but no blackening takes place unless the solution has free access to oxygen. If the red solution is boiled, immediate decolorisation takes place and subsequent exposure to the air causes the precipitation of melanin. In this way, by the use of colloidal ferric hydroxide to remove tyrosinase from the coloured solutions, Bach's theory [1914, p. 222] that tyrosinase contains a hydrolytic oxidation enzyme and that the conversion of the red substance into the colourless compound is an enzymic reduction process is disproved. It has also been found that the decolorisation of the red substance takes place spontaneously, though it may be accelerated by the enzyme, possibly by a process of adsorption. It takes place in the absence of oxygen, and can be quickened by raising the temperature, taking place extremely rapidly at 100°. These, and certain other facts suggest to us that the process is due to an intramolecular change.

In repeating the experiment of Bach [1914, p. 222] the red solution obtained by the action of 1 vol. of potato juice on 10 vols. of a saturated tyrosine solution at p_{H} 6.0, which contained tyrosine, tyrosinase, the red substance, and possibly some of the colourless compound, was divided into two parts. One half was treated with colloidal ferric hydroxide and filtered. A clear red solution was obtained which contained no enzymes. This filtrate and the other half of the original red solution containing tyrosinase were placed in flasks, the latter evacuated to remove all the oxygen, and then filled with CO_2 . They were then made airtight with rubber stoppers and placed in an incubator at 37°. After some time both solutions became colourless, but on shaking with air, only that containing tyrosinase became red again. Thus the subsequent reddening obtained by Bach, and attributed by him to the reoxidation of the colourless substance, is really due to the renewal of the action of tyrosinase on tyrosine, both of which are still present in the solution. This experiment shows clearly that the decolorisation of the red substance is not a process of reduction by the enzyme, for it takes place in the absence of the latter, and

also the colourless substance cannot be oxidised to the red compound again by shaking with air or oxygen.

Other evidence pointing to the non-existence of a hydrolytic oxidation enzyme in potato juice is our inability to obtain any oxidation of tyrosine by tyrosinase in the absence of oxygen and in the presence of methylene blue as a hydrogen acceptor. Buffered 0.05 % tyrosine solutions at p_H 6.0 and 8.0 with one-tenth their volume of potato juice or dialysed potato juice do not undergo any visible oxidation in an atmosphere of hydrogen or *in vacuo*, even if varying amounts of methylene blue are previously added. No reddening or darkening of the solutions occurs, even after 48 hours in an incubator, but subsequent admission of air causes fairly rapid oxidation, showing that the enzyme has not lost its activity towards tyrosine.

THE KINETICS OF THE TYROSINASE-TYROSINE REACTION.

In the following experiments, an attempt has been made to study this oxidation process on quantitative lines, to determine the "order" or type of the reaction, and also to make a careful study of the effects produced by adding to the enzyme certain constituents of potato juice which by themselves have no action on tyrosine. The results obtained indicate that the reaction is of the ordinary uni-molecular type and that the activity of the enzyme decreases very little during the reaction. Further, it is shown that in order to make any useful comparison, it is essential that the solutions used should be at the same hydrogen ion concentration, and should have an efficient buffering capacity. Thus we can confirm the statement of Chodat and Wyss [1922], that the results previously obtained by Haehn [1920] were simply due to alterations in the hydrogen ion concentration of the solution. In one respect, however, we have confirmed Haehn's results and find that potato juice rendered almost inactive by dialysis can be activated by the addition of certain mineral salts. This activation is quite different from that brought about by boiled potato juice, and our experiments show that the latter effect is not due to inorganic salts. It is found that certain samples of boiled potato juice, which are themselves inactive towards tyrosine, are able to activate the tyrosinase of potato juice, but the nature of this "co-enzyme" or activator is uncertain. This "co-enzyme" is relatively heat stable, but is not in the nature of an inorganic salt, because the ash of the same boiled potato juice has no activating power.

In order to follow quantitatively the relations existing between the enzyme and the substrate, only one of the two usual procedures has been used. Tyrosine being only slightly soluble in water, it is not possible to allow varying amounts of the enzyme to act upon a tyrosine solution and to assume that the concentration of the substrate remains constant during the experiment. In consequence, the rate of disappearance of tyrosine in the presence of a constant amount of enzyme has been followed. The method is laborious and time consuming, since the intermediate products of the oxidation as well as

the final product, melanin, have to be removed before the tyrosine can be estimated. A colorimetric estimation involving the use of a phosphotungstic and phosphomolybdic acid reagent, was found to have no advantage in this respect over the older method of bromination, so the latter was adopted. Errors due to bromine-absorbing compounds other than tyrosine, which were present in the enzyme solution, were eliminated by the use of controls.

General method of procedure. The method consisted of placing the tyrosine and control solutions contained in 500 cc. round-bottomed flasks in a thermostat at 20° and drawing through each solution a constant current of air saturated with water vapour and toluene. All the solutions were buffered by means of mixtures of NaOH and KH_2PO_4 solutions, and, by the addition of distilled water when necessary, it was arranged that all the solutions in comparative experiments should have the same total volume. In the case of the tyrosine solutions, 300 cc. of a phosphate buffered 0.05 % solution of tyrosine were used in each experiment, and for each of these solutions a control was set up, using the same quantity of distilled water and buffering solutions. Equal amounts of potato juice were added to all the solutions, together with 10 cc. of toluene, the latter preventing frothing and bacterial decomposition. The potato juice used was obtained by the process described above (p. 457). This juice was not purified in any way since the bromine absorbing capacity did not prove to be very high.

Samples (20 cc.) were withdrawn from each solution at the commencement of the reaction, and also after certain intervals. Each sample was immediately treated with 0.5 cc. of 10 % acetic acid and boiled to destroy the enzyme and coagulate the protein matter. After being plugged with cotton-wool, the flask containing the sample was allowed to stand for two days. The solution was then filtered, the precipitate of melanin and protein coagulum being washed several times with hot water to remove all the tyrosine adhering to the precipitate, and the filtrate made alkaline by the addition of 1 cc. of 10 % sodium carbonate. The solution was then boiled, the flask plugged with cotton-wool, and allowed to stand 24 hours. 1 cc. of 10 % acetic acid was then added, the solution boiled, allowed to stand for a few hours, and finally filtered. The tyrosine in each sample was then estimated by the method described below. This treatment of the samples was the one found most satisfactory in removing the intermediate products of oxidation together with most of the substances present in potato juice which absorb bromine. In spite of this very drastic treatment, however, the samples from the controls, *i.e.* the solutions containing only the buffering salts and potato juice, absorbed a small amount of bromine, but this remained almost constant throughout the experiment. Thus, by subtracting the "tyrosine figure" of the control from that of the corresponding tyrosine solution, a true "tyrosine figure" has been obtained for each sample and these enabled us to calculate the concentration of tyrosine in each solution after the various intervals of time.

These figures of tyrosine content have been used directly to draw curves

showing the relationship between the concentration of tyrosine and the time of reaction, and have also been used to calculate the values of k , the velocity coefficient, on the assumption that the reaction is of the uni-molecular type. The constancy of k throughout the experiments indicates that this assumption is correct, and thus the velocity of the reaction at any particular time is directly proportional to the concentration of tyrosine at that moment.

The estimation of tyrosine. The method, which is a modification of that used by Millar [1903], consists of adding to each tyrosine solution a standard solution of bromine containing more bromine than can be absorbed by the tyrosine and other bromine-absorbing substances present and estimating the excess.

The tyrosine solution was treated with 10 cc. of standard NaBrO_3 (0.8502 g. per litre; 1 cc. = 0.001527 g. tyrosine) solution, 2 cc. of 50 % KBr solution, and 7.5 cc. of 20 % HCl solution, the flask corked and allowed to stand 20 minutes. 2 cc. of 10 % KI solution were then added, and the free iodine titrated with $N/50 \text{ Na}_2\text{S}_2\text{O}_3$ solution. After making allowance for the bromine absorption of the control, the true tyrosine content was calculated as below, but in some cases the differences between the sodium thiosulphate titration figures of each sample and the corresponding control were used to represent the relative concentrations of tyrosine.

The $\text{Na}_2\text{S}_2\text{O}_3$ solution was first standardised by liberating a known amount of bromine from solutions of NaBrO_3 , KBr and HCl, replacing the free bromine by iodine, and titrating the latter with the thiosulphate solution.

All the following experiments were carried out in a thermostat regulated at 20° . A qualitative experiment showed that the rate of oxidation increased with rise of temperature.

Comparison of the influence of air and oxygen on the rate of oxidation. In all the experiments, a constant current of air was drawn through each solution during the whole of the reaction under observation, and thus it was first necessary to prove that the oxygen supply was adequate. If this were not so, the reaction followed would merely be the enzyme action controlled by a limited oxygen supply, and if the enzyme is of the ordinary oxidase type, that reaction would be the rate of formation of the peroxide.

The method adopted to prove this point before passing on to the more important investigations, was to compare the rate of oxidation under these conditions with the rate when the oxygen supply was increased five-fold by replacing the air current by an oxygen current approximately equal in volume.

Solution A. 300 cc. phosphate buffered 0.05 % tyrosine solution (p_{H} 8.0) + 30 cc. potato juice + 10 cc. toluene.

.. B. 300 cc. phosphate buffered 0.05 % tyrosine solution (p_{H} 8.0) + 30 cc. potato juice + 10 cc. toluene.

Control A. 300 cc. phosphate buffered water (p_{H} 8.0) + 30 cc. potato juice + 10 cc. toluene.

.. B. 300 cc.

Air was passed through A and Control A. Oxygen through B and Control B. "

Samples (20 cc.) of each of the four solutions were then taken at the commencement of the reaction, and after certain intervals, and after being treated

as indicated previously, the tyrosine in each was estimated by the bromination method.

Results. Amount of tyrosine (g.) present in the original volume of solution (330 cc.):

<i>A</i> (air)	0.1437	0.1275	0.0954	0.0716	0.0573
<i>B</i> (oxygen)	0.1437	0.1272	0.0935	0.0748	0.0574
Time (mins.)	0	67	160	250	315

The control figures remained constant, or the variations were within the limits of experimental error, and both controls gave the same titration figures.

The above results prove conclusively that the oxygen supply is adequate if a current of air similar to that used in this experiment is drawn through each solution and if this supply is maintained throughout the experiment. It may also be noted that this current of air also keeps the solution well agitated and so prevents differences in temperature or local concentration of enzyme.

Influence of hydron concentration on the velocity of oxidation. In this experiment, three tyrosine solutions (*D*, *C* and *B*) were prepared, buffered by phosphate solutions at p_{H} 6.0, p_{H} 7.0 and p_{H} 8.0 respectively, and a control (*A*) was set up, using the same amount of phosphate solution at p_{H} 6.0. A preliminary experiment proved that controls at the three different hydron concentrations gave equal thiosulphate titration figures, and so only one control was necessary for this comparative experiment. To 300 cc. of each of the solutions *B*, *C*, and *D* and solution *A* 30 cc. of potato juice were added and 10 cc. of toluene, and they were then placed in the thermostat at 20°. The same current of air was passed through all four solutions. Duplicate samples were withdrawn at intervals and treated as described above.

Results. Grams of tyrosine in 330 cc.:

<i>B</i> , p_{H} 8.0	0.1198	0.0911	0.0781	0.0706	0.0629	0.0561	0.0148
<i>C</i> , p_{H} 7.0	0.1179	0.0973	0.0821	0.0772	0.0688	0.0603	0.0214
<i>D</i> , p_{H} 6.0	0.1168	0.0963	0.0855	0.0796	0.0730	0.0622	0.0339
Time (mins.)	0	135	233	313	418	485	1420

Velocity coefficients. These were calculated on the assumption that the reaction is uni-molecular, *i.e.* $k = \frac{1}{t} \log_e \left(\frac{a}{a-x} \right)$ or $k = \frac{2.302}{t} \log_{10} \left(\frac{a}{a-x} \right)$, where a = the original concentration of tyrosine and $(a - x)$ the concentration after time t .

Velocity coefficients (k).

							Average
<i>B</i> , p_{H} 8.0	0.0020	0.0018	0.0017	0.00155	0.0016	0.0015	0.0017
<i>C</i> , p_{H} 7.0	0.0014	0.0015	0.0014	0.0013	0.0014	0.0012	0.0014
<i>D</i> , p_{H} 6.0	0.0014	0.0013	0.0012	0.0011	0.0013	0.0009	0.0012
Time (mins.)	135	233	313	418	485	1420	—

This experiment was repeated later with another enzyme preparation, and the following figures obtained:

Velocity coefficients (k).

						Average
p_{H} 8.0	0.0023	0.0023	0.0022	0.0019	0.0022	
p_{H} 7.0	0.0015	0.0017	0.0020	0.0019	0.0018	
p_{H} 6.0	0.0015	0.0015	0.0015	0.0013	0.0015	
Time (mins.)	66	188	375	1440	—	

The curves obtained by plotting the concentration of tyrosine against the time are of the true logarithmic type, indicating that the amount of tyrosine oxidised during any short interval of time is directly proportional to the average concentration of tyrosine during that interval. In Fig. 1 the curve at p_{H} 8.0 was obtained by substituting for k in the formula $k = \frac{1}{t} \log_e \frac{a}{a-x}$, the average value for k found in the experiment at p_{H} 8.0, and calculating from this the corresponding values of $(a-x)$ or x for different intervals of time. Points are also marked on the graph which represent the concentrations of tyrosine actually found by estimation, and it will be seen that these are in close agreement with the average curve. Similar curves are also drawn giving the oxidations at p_{H} 6.0 and p_{H} 7.0, and it will be observed that the velocity is greatest at p_{H} 8.0 and least at p_{H} 6.0.

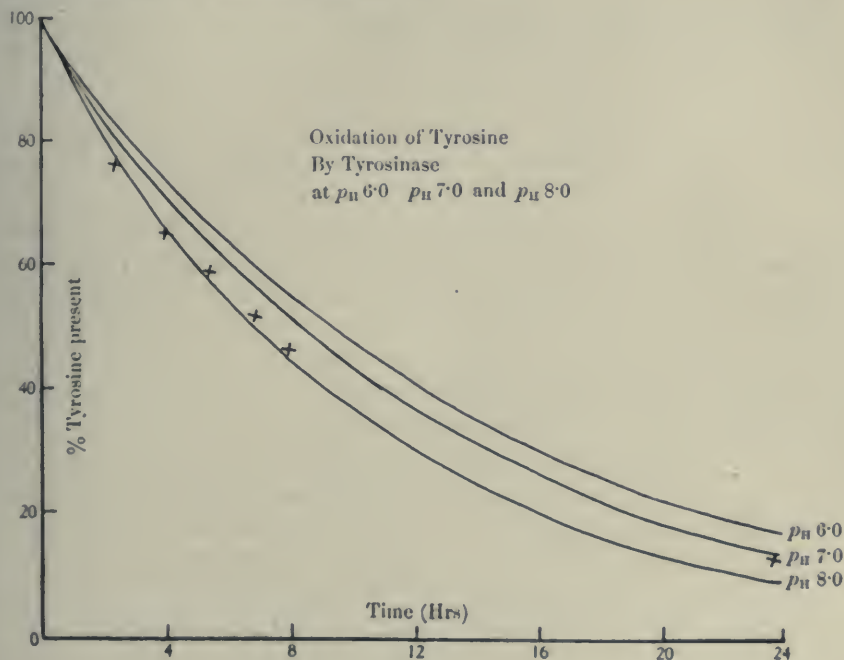


Fig. 1.

x — Experimental points for p_{H} 8.0.

The approximate constancy of the values of the velocity coefficient during the whole of the reaction proves that the reaction is of the uni-molecular type and is therefore comparable with other enzyme actions. In this constancy of the values of k , however, the reaction seems to deviate somewhat from those followed by other workers. Thus in the case of the hydrolysis of milk-sugar by lactase [Armstrong, 1904] the value of the velocity coefficient falls rapidly from 0.0640 for the first hour to 0.0310 for 5 hours and 0.0129 for 24 hours. In the case of the hydrolysis of cane-sugar by invertase [Henri, 1903] the velocity coefficient steadily increases from 0.00058 during the first

hour to 0.00085 during 11 hours. This constancy in the case of the tyrosinase-tyrosine reaction is probably due to the absence or elimination of certain factors which would have an accelerating or retarding effect on the reaction. Thus the nature of the reaction seems to preclude the possibility of a reverse reaction playing any part in the process, while effects due to changes in hydron concentration may be ruled out on account of the buffering of the solution. With regard to the destruction or change in the nature of the enzyme, our observations lead to the conclusion that tyrosinase is not very unstable at these hydron concentrations.

The values obtained for the velocity coefficient fluctuate during the experiment or show a slight decrease during the oxidation of half the tyrosine, followed by a larger decrease during the oxidation of the remainder. These divergences during the first six or eight hours are not very great, except perhaps at p_{H} 8.0 and in view of the small amount of tyrosine present in each sample (0.0075 g. in the first samples and about 0.0010 g. in the last) and the rigorous treatment to which these samples must be submitted, these divergences are not greater than the experimental error involved in their determination. With reference to the "slowing" which occurs during the second part of the reaction, it is interesting to note that this is less marked in the experiments carried out at p_{H} 7.0 and this indicates certain factors which may be the cause of retardation. In acid solutions, the enzyme is precipitated, and although this is not very marked at p_{H} 6.0, it will have an appreciable effect on reactions proceeding for 24 hours, and will cause a decrease in velocity similar to that observed. With regard to the decrease at p_{H} 8.0, no destruction or precipitation of the enzyme should take place, because tyrosinase seems to be very stable and well dispersed at this hydron concentration, and the determining factor appears likely to be the adsorption of the catalyst by the melanin produced, this being the main product of oxidation under these conditions.

The results obtained indicate that slight alkalinity favours the action of the enzyme, for the velocity at p_{H} 8.0 is greater than that at p_{H} 7.0, and this in turn is greater than that at p_{H} 6.0. The exact conditions determining this greater velocity at p_{H} 8.0 are not known, but it is suggested that there are two factors which probably play a very important part in this phenomenon. Firstly, the dispersion of the enzyme, as judged by the naked eye, is best at this hydron concentration, and secondly, if the first reaction, the conversion of tyrosine into the red pigment, tends to be at all reversible, this will have least effect at p_{H} 8.0, because of the rapid conversion of the intermediate products into the final product, melanin.

THE EFFECT OF ADDING BOILED POTATO JUICE.

Some ordinary potato juice was boiled in a small Erlenmeyer flask for 2-3 minutes, cooled and filtered. When tested with a tyrosine solution, it did not give the characteristic tyrosinase reaction, showing that the enzyme present had been destroyed.

- A. 300 cc. phosphate buffered 0.05 % tyrosine solution (p_{H} 8.0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.
- B. 300 cc. phosphate buffered 0.05 % tyrosine solution (p_{H} 8.0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.
- Control A. 300 cc. phosphate buffered water (p_{H} 8.0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.
- „ B. 300 cc. phosphate buffered water (p_{H} 8.0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

Results.

Tyrosine present in g. per 330 cc. (Average of two estimations.)

	1	2	3	4	5
A. Ordinary potato juice	0.1427	0.1269	0.1062	0.0905	0.0321
B. Potato juice and boiled potato juice	0.1442	0.1185	0.0980	0.0804	0.0287
Time (mins.)	0	130	220	360	1380

The comparison is easier if the amount of tyrosine oxidised is represented as a percentage of the amount originally present, *i.e.* % of tyrosine oxidised.

	1	2	3	4	5
A.	0	11.08	25.58	36.59	77.50
B.	0	17.82	32.03	44.24	80.09

Average velocity coefficient for A = 0.00114.

„ „ B = 0.00151.

This activation has been noticed by Haehn [1919] who used boiled potato juice and tyrosine freed from the non-colloidal matter of potato juice by ultrafiltration, but this author erroneously attributes it to the presence of certain salts (calcium salts, phosphates, etc.) in the potato juice which act as co-enzymes. This activator or co-enzyme is not an ordinary inorganic salt, but is probably of organic nature, since it is not present in the ash of boiled potato juice, as shown below. It is interesting to note in this connection, that this activator is nearly always present in the boiled juice of new potatoes, but rarely, if ever, present in that from old potatoes.

EFFECT OF ADDING THE ASH OF BOILED POTATO JUICE.

Boiled potato juice containing the activating agent referred to in the previous experiment, but having no tyrosinase reaction, was evaporated down to dryness and ashed. The ash was dissolved in a little warm dilute HCl, neutralised and made up to the original volume, a precipitate of the hydroxides of aluminium and iron remaining in suspension. To prove the activating power of the original boiled potato juice, an experiment was included to show that the oxidation of tyrosine by the unboiled potato juice used was accelerated by the addition of some of this boiled juice.

- Solution A. 300 cc. tyrosine solution (buffered at p_{H} 8.0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.
- „ B. 300 cc. tyrosine solution (buffered at p_{H} 8.0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.
- „ C. 300 cc. tyrosine solution (buffered at p_{H} 8.0) + 15 cc. potato juice + 30 cc. solution of ashed juice + 10 cc. toluene.

Controls *D*, *E* and *F* for solutions *A*, *B* and *C* respectively were set up under exactly the same conditions, except that the buffered solutions in these cases contained no tyrosine.

Results.

Time (mins.)	0	128	243	385										
	Tyrosine (g.) present in 330 cc. of solution. Average of 2 estimations				% tyrosine oxidised			Velocity coefficients						
	1	2	3	4	2	3	4	2	3	4	Average			
A. (normal)	0.1415	0.1237	0.1050	0.0878	12.58	25.80	37.95	0.0011	0.0012	0.0012	0.0012			
B. (+ boiled juice)	0.1415	0.1144	0.0938	0.0747	19.19	33.69	47.23	0.0017	0.0017	0.0017	0.0017			
C. (+ ashed boiled juice)	0.1415	0.1248	0.1066	0.0896	11.73	24.64	36.67	0.0010	0.0012	0.0012	0.0011			

SUMMARY AND CONCLUSIONS.

1. Tyrosinase first produces from tyrosine a red substance. This process requires the presence of the enzyme and only takes place in the presence of oxygen. This red substance becomes colourless spontaneously and is then oxidised to form melanin. These last two processes take place in the absence of tyrosinase, but may be accelerated by it or by other oxidases found in potato juice.

2. The hydrion concentration of the medium has a marked influence on the velocity of the oxidation of tyrosine by tyrosinase and an even greater influence on the nature of the pigments produced. The limits of p_{H} between which potato tyrosinase acts upon tyrosine are 5 and 10. In the neutral and acid solutions, the main product during the first six hours is the red substance, but in alkaline solutions, the conversion of this into melanin is so rapid that the solutions quickly darken, and the preliminary reddening is not very marked.

3. Evidence is presented which is opposed to the view of Bach that tyrosinase contains a hydrolytic oxidation catalyst.

4. Haehn's experiment showing that tyrosinase, which has been rendered relatively inactive by dialysis, can be activated by the addition of certain mineral salts has been confirmed. But the acceleration of the enzyme action brought about by the addition of boiled potato juice is not due to inorganic salts but to some organic constituent or constituents of the potato juice. This activator is not always present in boiled potato juice, but is usually to be found in the boiled juice of new potatoes.

5. In order to obtain any true comparison between the oxidations of tyrosine by tyrosinase under different conditions, it is necessary to have all solutions well buffered at a definite hydrion concentration. In addition, the temperature must be kept constant and a satisfactory supply of oxygen must be maintained.

6. The results obtained show that the reaction is of the uni-molecular type, giving a true logarithmic curve when values of the concentration of tyrosine are plotted against the time, and also giving values for the velocity coefficient, which are approximately constant throughout the reaction. Tyrosinase is thus a true catalyst.

7. The oxidation of tyrosine by tyrosinase in a buffered solution at p_{H} 8.0 has a greater velocity than that at p_{H} 7.0, and the velocity at p_{H} 7.0 is greater than that at p_{H} 6.0.

8. The acceleration of the enzyme action by the addition of boiled potato juice has been definitely proved by following the rate of disappearance of tyrosine and it has also been shown that the ash of the same boiled juice produces no acceleration of the action.

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LVI. THE ISOLATION FROM CABBAGE LEAVES OF A CARBOHYDRATE, HITHERTO UNDESCRIBED, CONTAINING THREE CARBON ATOMS.

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THE substance described in the following paper was obtained during the investigation of the water-soluble constituents of cabbage leaves. The main object of the research was the isolation of the simpler nitrogenous substances present, but non-nitrogenous substances obtained were also examined, it being thought that these might throw some light on the derivation of the nitrogenous bodies.

During the course of the work, the method for separation of amino-acids and peptides from sugars by the carbamate method [Buston and Schryver, 1921] has been elaborated and rendered more complete; fractions containing 8-12 % nitrogen have been produced, which await further investigation. Amongst non-nitrogenous substances isolated may be mentioned citric and mesaconic acids. The latter does not appear so far to have been isolated as a natural product, and its preparation from cabbages may throw some light on the formation of citric acid from glucose. These points will form the subject of a later communication.

The product described in this paper appears to be of sufficient interest to warrant a separate mention.

The method adopted was briefly as follows:

An ether-water extract of 90 kilos. of the minced leaves was obtained, and the protein present flocculated as described by Chibnall and Schryver [1921]. The clear solution was then precipitated successively with alcohol, phosphotungstic acid, and baryta-alcohol; finally the major part of the nitrogenous substances was removed by repeated treatments with baryta and carbon dioxide in the presence of alcohol, according to the method described by the authors [1921].

The liquid remaining after this treatment was treated with sulphuric acid to remove barium quantitatively, and evaporated to a syrup. The syrup was left standing for two or three days under three times its weight of 90 % alcohol, by which time a fair quantity of crystalline matter had separated. This was sucked off on the pump, and dried on a porous plate. 36 g. of pale brown, somewhat sticky crystals were obtained, containing 0.9 % nitrogen. By treating in a Soxhlet for six hours with absolute alcohol, the colouring

matter and nitrogenous substances were removed, leaving 25 g. of white, crystalline material. Of this, 51 % was found to be inorganic matter, mainly potassium chloride. The crystals were boiled with glacial acetic acid and filtered hot; on cooling, a bulky crop of fine white needles separated. These were removed and dried on a plate; weight obtained, 9.5 g. The mother liquor, on evaporation, gave a further crop of 1.2 g. The crystals still contained small amounts of potassium chloride, but were freed from this by repeated recrystallisation from glacial acetic acid. The total yield was about 10 g. from 90 kilos. of leaves.

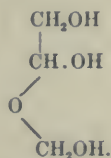
The substance was finally obtained as a white crystalline powder, readily soluble in water and in hot glacial acetic acid; but practically insoluble in alcohol and other organic solvents. The substance possessed a faint but perceptible sweetish taste. Its melting point was 148° .

The combustions gave the following figures:

C = 33.44, 33.19 %; H = 7.30, 7.49 %; O = 59.26, 59.32 %.

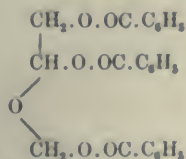
A molecular weight determination in aqueous solution by the freezing point method gave: $M = 111.4$. The above figures indicate the formula to be $C_3H_8O_4$. (This requires C = 33.34 %; H = 7.40 %; O = 59.26 %. Mol. wt. 108.)

The only constitutional formula to satisfy this would be that of a trihydroxy-ether:



To test this, it was decided to prepare the benzoyl derivative. 0.3 g. of the substance, treated in the ordinary way with benzoyl chloride and alkali, and extracted with ether, gave, on evaporation of the ether solution, 1.3 g. of a colourless, almost solid syrup. This was granulated with ligroin and could then be recrystallised from benzene. 0.7 g. white crystals were thus obtained; m.p. 52° – 53° . Combustion of this gave: C = 68.20 %, H = 5.11 %, O = 26.69 %. Molecular weight in benzene solution, 434. (The tri-benzoyl derivative requires C. 68.58 %; H = 4.78 %; O = 26.68 %. Mol. wt. 420.)

The benzoyl derivative must therefore contain three benzoyl groups, and have the formula:

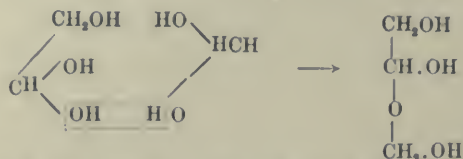


So that the original substance must contain three hydroxy groups, and have the formula given above.

Examination of this formula will show that the central carbon atom is asymmetric; the substance obtained, however, was optically inactive. This

may have been due to racemisation produced by the hot glacial acetic acid, or by the long contact with baryta in the earlier stages of separation.

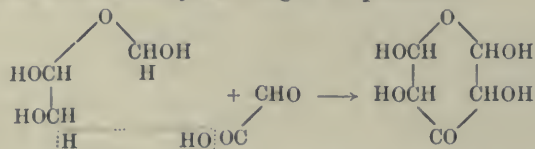
The formula also suggests the derivation of the substance by the condensation of formaldehyde and glycollic aldehyde (which may itself be regarded as the first condensation product of formaldehyde), reacting in the hydrated form:



It might thus be regarded as a simple disaccharide, glycollic aldehyde and formaldehyde being regarded as diose and monose respectively. Its formation from this source would thus make it appear to be formed as an alternative to a triose.

Various experiments were carried out to test for disaccharide-like properties in the substance. It showed no reducing properties, and gave no osazone. After heating with 5–10 % hydrochloric acid for some time, the neutralised solution still did not reduce Fehling's solution, though it reduced permanganate on warming. No formaldehyde could be detected. The substance therefore differs from normal disaccharides in its great stability (resembling more the ethers, in which class its formula also places it). Possibly the smallness of the molecule is the cause of its stability.

It is interesting to note further that by condensation with a molecule of glyoxylic acid a pyrone ring, which forms the basis of many naturally occurring substances such as the anthocyanins, might be produced:



Various similar condensations of the substances or its derivatives could be suggested.

SUMMARY.

An account is given of the isolation from cabbage leaves of a carbohydrate hitherto undescribed, of the formula $\text{C}_3\text{H}_8\text{O}_4$. This crystallises in needles, m.p. 148° which are readily soluble in cold water and hot glacial acetic acid, but insoluble in most other organic solvents. It yields a tri-benzoyl derivative, formula $\text{C}_3\text{H}_5\text{O}_4(\text{C}_6\text{H}_5\text{CO})_3$; m.p. 52° – 53° . The carbohydrate therefore has the constitution $\text{CH}_2(\text{OH}) \cdot \text{CH}(\text{OH}) \cdot \text{O} \cdot \text{CH}_2(\text{OH})$.

A possible mode of formation from formaldehyde in the plant is outlined, and it is suggested that it may form a stage in the making of a pyrone ring, which is an important constituent of the molecules of the anthocyanins.

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LVII. INVESTIGATIONS ON GELATIN. PART II. RESEARCHES ON THE METHODS OF PURIFYING GELATIN.

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*Report to the Adhesives Committee of the Department
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INTRODUCTION.

IN spite of the fact that a very large literature exists dealing with the physical and chemical properties of gelatin, no definite criterion seems to have been established up to the present time by means of which the purity of the material investigated can be defined. The necessity for defining the state of purity is an urgent one, when the origin of a sample of gelatin is considered. In the first place, it is derived from animal tissues, which, in addition to the actual precursors of gelatin, contain other complex nitrogenous substances, which may contaminate the finished product, and in the second place, it is itself produced from these precursors by the chemical action of hot water, undergoing during the process degradation into simpler products, the nature of which has not yet been definitely established. It follows, therefore, that every sample of gelatin is a mixture of gelatin with other nitrogenous substances, even when the greatest care has been taken in its preparation.

The following investigations were undertaken with two main objects in view. It was desired in the first instance to establish some physical criterion, by means of which the state of purity of a given sample could be defined; in the second instance it was necessary to discover processes by means of which the nitrogenous contaminations of gelatin could be removed, so as to yield a product conforming with the established physical criterion, which should remain unchanged when these processes were repeated an indefinite number of times.

An ordinary high grade commercial preparation, when allowed to soak in water, yields to the aqueous phase certain amounts of diffusible nitrogenous substances, the amounts of which vary from preparation to preparation, when the same conditions of experiment are chosen. These substances comprise, for the most part, the contaminating substances to which reference has been

already made. If a gelatin preparation is free from these, then a definite equilibrium of some kind should exist between the nitrogenous contents of an aqueous phase and a gelatin phase (either in the form of a jelly or otherwise) which are in contact with one another. If this equilibrium can be ascertained, then a criterion for the definition of the state of purity of a sample of gelatin will have been established.

As a result of the investigations described in this communication, preparations of gelatin have been obtained in which the desired nitrogenous equilibrium has been found to exist, and which must be taken, therefore, to be free from diffusible nitrogenous contaminations.

The researches started with the determination of the amount of nitrogen which diffused out from a given surface of a gelatin jelly, prepared under certain specified conditions, into a given volume of water in contact with it within 20 hours at the temperature of 20°. This method of investigation yielded valuable information as to the character of commercial samples of gelatin and glues (to which reference has been made in the *Bulletin on Adhesives* issued by the Department of Scientific and Industrial Research in 1922) and the determination of the so-called "diffusible nitrogen" is suggested as a routine method for the examination of these products. If the water in contact with the jelly is removed daily, and the nitrogen is estimated in each portion, it will be found that this diminishes rapidly until it reaches a small quantity, which remains constant over a prolonged period. Similar experiments were carried out with relatively large amounts of gelatin jelly with large surfaces in contact with water, and it was found that even after several months of contact, the amount of nitrogen in the water phase was continually increasing. The experiments illustrating these statements are given in Section I of this paper.

A detailed examination was then undertaken of certain properties of the nitrogenous diffusate, and also of a solution of gelatin (10 %) which had been heated for several hours, as it was assumed that the diffusate consisted largely of the products of thorough decomposition of gelatin. When these products were submitted to dialysis through parchment paper, it was found that at first relatively large amounts of nitrogenous substances diffused through in one day, but these somewhat rapidly diminished, until, after a certain time, the quantities passing the dialyser in a day became practically constant. This apparent constancy was maintained for several weeks. A diminution of the rate of diffusion could then only be ascertained when the diffusate of several days was estimated together. The rate of diffusion was so slow, that the results originally suggested that there were products present within the dialysers which were undergoing continual degradation in the presence of water at ordinary room temperature. These results are described in Section II.

The general result of the experiments given in Sections I and II was to indicate the great difficulty, if not the impossibility of freeing gelatin from the nitrogenous substances contaminating it by the method of washing or dialysis.

Incidentally, an examination was made of the products which passed the dialysers and those which remained within. The differences in the "Hausmann numbers" indicate that gelatin on heating does not break down into smaller aggregates of the same chemical character. The nature of the thermal decomposition of gelatin has not yet been definitely established.

As it had been shown that gelatin could not be freed from the contaminating nitrogenous substances even by months of continuous washing and dialysis, the effect of electrolysis on their removal was next investigated. It was found that by this means simple electrolytes such as salts were readily removed. Electrolytes of colloidal character and non-electrolytes remained, however, in the gelatin, when submitted to electrolysis. A simple ampholyte, such as glycine, which has weak acidic and basic dissociation constants, was removed only very slowly under the influence of an electric current. The result of these experiments, which are described in Section III, was to indicate, that, even by electrolysis, the nitrogenous contaminating products could not be completely removed.

Electrolysis, however, removes very readily the last traces of salts and other strong electrolytes from gelatin, which can thus be obtained practically ash-free. The electrolysis process, as described in this paper, is best applied to gelatin, from which the greater part of the salts have been already removed by washing the jellies with dilute acid and water.

When freed from electrolytes, gelatin possesses the property of separating out as a hydrated insoluble precipitate when solutions of 2 % concentration or less are cooled. Advantage has been taken of this fact to purify gelatin still further by a process conveniently termed "recrystallisation." The nitrogen content of the clear liquid after separation of the insoluble hydrate has been determined after successive "recrystallisations." This was found to fall to a certain constant value which remained the same whether the gelatin separated from a concentration of 0.1 or 2 %. Such a condition should hold when a substance is in equilibrium with its saturated solution, and the actual experiments indicate that such a saturated solution contains about 0.056 % of gelatin¹. This result is in close accord with that found by Fairbrother and Swan [1922] although obtained by an entirely different method. Some of the results of these investigators are not, however, in agreement with those obtained by the authors of the present paper. It has been found impossible to obtain nitrogenous equilibrium between the aqueous phase and the gelatin phase in the short time suggested by Fairbrother and Swan; such an equilibrium was not attained with a well-washed gelatin sample even after several months. The gelatin was estimated by those authors in the aqueous phase by precipitation with tannic acid. This reagent will, however, precipitate, in addition to gelatin, a great part of the degradation products which are present as contaminants. The method employed by Fairbrother and Swan appears to the authors of this paper to be affected by two sources of error, which will

¹ See however, p. 486.

tend to compensate one another, and the accordance found in the determination of the solubility of gelatin in water by the two sets of investigators must be to a large extent accidental. The experiments on the "recrystallisation" of gelatin are described in Section IV.

According to the researches herein described, gelatin free from soluble nitrogenous contaminants can be obtained by submitting it to the following successive processes: (i) washing the jelly first with weak acid and then with water, (ii) submitting the washed concentrated jelly to electrolysis, and (iii) recrystallising the electrolysed gelatin.

Certain samples of gelatin thus purified still appear to contain a very small amount of an insoluble nitrogenous contaminant, which imparts to the jellies an opalescent appearance. Investigations on the separation of this are still in progress. Some gelatins appear to be nearly free from it. It is, however, only present in very small quantities and does not influence to any large degree the properties of gelatin, as do the water-soluble contaminants.

EXPERIMENTAL.

SECTION I. THE "DIFFUSIBLE NITROGEN."

When gelatin is placed in contact with water, a certain amount of nitrogenous material passes out from it. If the experiment is carried out under conditions defined as regards strength of gelatin, volume of gelatin and water, area of contact of phases, time of contact and temperature, the number of milligrams of nitrogen which pass into solution is termed the "diffusible nitrogen."

The conditions employed in the experiments described below were as follows: 20 g. of gelatin and 100 cc. of water were placed in a wide-mouthed stoppered bottle of $9\frac{1}{2}$ cm. internal diameter. The gelatin was dissolved in the water at 37° , and the solution allowed to set for 24 hours at room temperature. 100 cc. of water were then poured gently on to the surface of the gelatin, and the bottle was placed in a thermostat at 20° for 24 hours. The water was then poured off through a filter, and the surface of the gelatin and sides of the bottle were washed once or twice with distilled water, which was also poured through the filter. The nitrogen in the filtrate was then determined. (The "diffusible nitrogen.") In nearly all the experiments described in this paper, Coignet's "gold-label" gelatin was employed. In a few experiments other samples of gelatin were also occasionally employed, and these yielded the same results as Coignet's. For the latter, the "diffusible nitrogen," determined under the conditions mentioned above, gave values varying from 10–20 mg. If, after the first determination of the diffusible nitrogen, the process is repeated on the same gelatin by the addition each time of 100 cc. of water, the value falls to 2 mg. per 24 hours, at which it remains sensibly constant (with occasionally higher values, owing to the swelling and increase

of surface of the gelatin). As an illustration, the results of one experiment are given below:

"Diffusible nitrogen" in Coignet's "gold-label" gelatin.

	Period of contact	Diffusible nitrogen mg.	Diffusible nitrogen in 24 hours mg.
1.	24 hours	11.6	11.6
2.	24 "	5.8	5.8
3.	48 "	8.65	4.32
4.	48 "	7.01	3.50
5.	48 "	6.46	3.23
6.	72 "	9.16	3.05
7.	8 days	20.6	2.57
8.	72 hours	9.37	3.12

Fresh water was used in each experiment.

The process cannot be carried on indefinitely, since the gelatin ultimately swells and the surface breaks up. If the gelatin is washed until the approximately constant nitrogen value is reached, and then remelted at 37°, and allowed to set, it is found that the "diffusible nitrogen" has risen again nearly to its original value. For example, a sample giving the initial value of 15.3 was washed till the value fell to 1.96. On remelting it rose to 12.1. It appears probable that the diffusible nitrogen comes from the surface layer or its immediate neighbourhood only. This statement is confirmed by experiments in which different areas of contact between jelly and water were used, the other conditions being exactly similar. Thus, when the ratio of the two surfaces was as 2.2:1, the diffusible nitrogens were 27.2 and 14.

The ultimate small value (2 to 3) obtained by continuous washing may be due to (a) simple solubility, (b) slow diffusion from the lower layers, (c) slow degradation of the gelatin in contact with water. The "diffusible nitrogen" is not exactly proportional to the concentration of the gelatin. Thus

	Concentration of gelatin	"Diffusible nitrogen"
	5 g. gelatin + 100 cc. water	7.4
10	" "	10.0
20	" "	12.6

Washing the gelatin with dilute hydrochloric acid and then with water (Loeb) produced a marked lowering of the diffusible nitrogen; thus, for example:

First	24 hours.	Diffusible nitrogen	2.5
Second	"	"	1.3
Third	"	"	1.25

Fresh water was used each time.

This case was somewhat exceptional, other samples giving the higher values of 4 to 6 for the first 24 hours.

The use of very dilute hydrochloric acid ($N/1000$ or less) for making up the gelatin and washing it makes no appreciable difference to the diffusible

nitrogen. The presence of neutral salts, on the other hand, increases it, as the following experiments (some of which were carried out by Mrs Horne) show:

Salt used	Concentration of salt in jelly and water	First four days	Next six days	Next eight days
NaCl	0.0	15.3	12.3	17.4
	N/10	18.6	14.7	20.3
	N/4	19.2	15.8	21.7
	N/2	21.9	18.2	23.3
CaCl ₂	0.0	17.4	15.6	16.5
	N/10	21.5	19.4	19.7
	N/4	24.9	—	19.5
	N/2	25.8	—	20.3

Fresh salt solutions were employed for each of the periods of experiment given in the above table.

The "diffusible nitrogen" is derived to a large extent, if not wholly, from the products of thermal degradation of gelatin, as is shown from the following experiment. A 20 % gelatin was made up in a series of wide-mouthed bottles, as described above, and each bottle was heated for a given period in a hot-water oven. After removal, the gelatin was allowed to set for at least 24 hours, and the "diffusible nitrogen" determined in the usual manner. The results are indicated in the accompanying table and curve (Fig. 1). After 16 hours the jelly is so weak, that the determination of the diffusible nitrogen is unreliable. After 29 hours' heating the gelatin would not set at all. The curve for the first 16 hours is a regular one.

The experiments were carried out with a gelatin that had been well-washed beforehand.

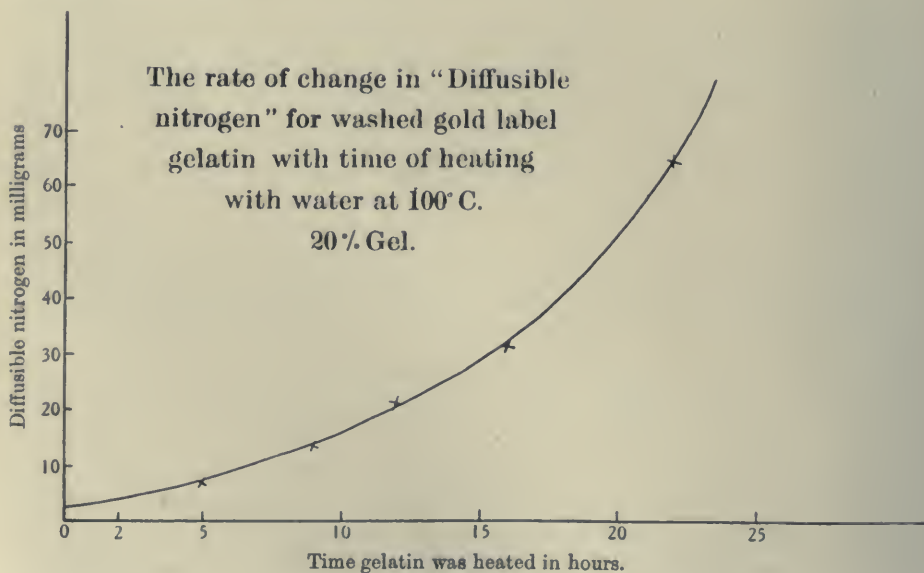


Fig. 1

		Diffusible nitrogen
Unheated gelatin		1.9
Heated 5 hours		6.9
" 9 "		13.1
" 12 "		21.4
" 16 "		32.6
" 22 "		59.9
" 29 "		Gelatin did not set

The question next arose as to whether, on leaving gelatin a sufficiently long time in contact with water, an equilibrium value for the nitrogen content of the water could be reached. To test this 112 g. of gelatin, previously washed carefully with dilute hydrochloric acid and water, were immersed in the form of thin sheets in 3 litres of water, a little chloroform having been added to keep the gelatin sterile. The whole was kept in a tightly stoppered bottle and left for prolonged periods during which, at intervals, 50 to 100 cc. of the aqueous phase were removed, filtered and submitted to an estimation of nitrogen. No indication of an equilibrium was ever found, as the following numbers indicate:

Period of contact	Milligrams of nitrogen per 100 cc. of aqueous phase
2 months	43.0
3 "	45.5
4 "	48.9

The water after this time was poured off, the gelatin was washed, and fresh water was added. The nitrogen in portions of 50–100 cc. was determined at intervals with the following results:

Period of contact	Milligrams of N per 100 cc. aqueous phase
7 days	11.2
14 "	12.6
21 "	14.6
28 "	16.0
53 "	20.3
4 months	35.3

Thus, after even 8 months of washing, nitrogen appeared to be still coming out from the gelatin into the water. These results are at variance with those of Fairbrother and Swan [1922]. They suggest that gelatin itself undergoes some degradation in contact with water. It was therefore considered advisable to obtain some idea as to the diffusibility of the nitrogenous products which could be washed out of the gelatin, and experiments dealing with this factor are given in the next section.

SECTION II. THE DIFFUSIBILITY THROUGH PARCHMENT OF THE NITROGENOUS PRODUCTS WHICH CAN BE EXTRACTED FROM GELATIN BY WATER.

Two main series of experiments were carried out. (a) With the washings of gelatin. (b) With a sample of gelatin, which had been previously well washed till nearly salt-free and then heated for a long period, so that it no longer set in concentrated solutions.

(a) *Experiment with the washings.* The following experiments carried out by Mrs Horne, were made with a sample of Nelson Dale and Co.'s bone gelatin, which was kindly provided by Mr W. K. Beveridge. Similar results were got with Coignet's "gold-label" gelatin.

Three extracts (I, II and III) were made. Extract I was made by extracting 2 lbs. of gelatin first with 10 litres of water for 4 days, then after pouring off the water which was not absorbed by the gelatin, and which amounted to about $2\frac{1}{2}$ litres, with 10 litres of water for 2 days, this extraction being followed by another extraction by 10 litres of water for 2 days. The washings from these extractions, which lasted altogether for 8 days, were combined to form extract I.

The second extract (II) was made by treating the washed gelatin with 10 litres of water for 13 days, whilst extract III was made by a subsequent extraction with the same amount of water for 24 days. The three extracts were concentrated under diminished pressure until they all contained the same amount of nitrogen, viz. 246.6 mg. per 100 cc. The concentrated solutions were then introduced into parchment dialysers and submitted to dialysis under as nearly as possible identical conditions, *i.e.* the size of the parchment bags, the size of the containing vessels, the amounts introduced into the dialysers, and the water outside were the same in all cases. All the experiments were carried out under bell-jars under which were placed also open dishes containing chloroform so as to keep the solutions sterile throughout the whole course of the experiments. 144 cc. of solution were introduced into the dialysers and 600 cc. of water were placed outside. The exterior water was changed from time to time and the nitrogen content thereof estimated. The period of dialysis into the same exterior liquid (600 cc.) is indicated in the second column.

*Rate of dialysis of nitrogenous substance from three washings
of the same portion of gelatin.*

Time from commencement of experiment Days	Period of dialysis Hours	Extract I mg. N	Extract II mg. N	Extract III mg. N
1	24	29.07	16.88	13.45
2	"	15.01	9.2	10.26
3	"	10.53	5.93	6.21
4	"	9.09	5.64	4.56
7	72	16.5	8.25	9.22
8	24	5.65	3.36	3.06
9	"	4.95	2.83	2.74
10	"	5.54	3.4	—
11	"	4.49	2.94	—
14	72	9.9	6.6	—
15	24	4.04	3.36	—
16	"	4.04	3.25	2.6
17	"	—	3.2	2.59
18	"	4.34	3.28	2.59
24	144	15.5	11.3	8.89
30	"	14.2	11.84	—
36	"	12.28	9.95	7.2
42	"	7.79	7.22	5.88

It will be noticed from the above table that even the third extract of the gelatin, after as long an interval of dialysis as 6 weeks, still contains nitrogenous substances which can pass the dialyser, the amount of nitrogen thus passing amounting to the quite appreciable quantity of nearly 1 mg. per day. About 30 % of the nitrogen of extract I and 18 % of extract II had diffused through the parchment in 14 days.

(b) *Experiment with heated gelatin solution.* 200 g. of gelatin, which had been previously washed till it was nearly ash-free, were dissolved in 4 litres of water, and the solution was heated for 48 hours in an enamelled iron digester with a reflux condenser in a glycerol bath at 110° . A dark-coloured insoluble compound was formed, which was filtered off. The filtrate was concentrated to a thick syrup under reduced pressure at 40° . This syrup was then dissolved in water and 200 cc. of a solution containing 1200 mg. of nitrogen were introduced into a parchment dialyser. The water on the outside of the dialyser was 500 cc. and this was changed daily and the nitrogen estimated therein. The results are given in the accompanying table. The second column of the table gives the amount of nitrogen in each sample of the dialysate, and the third column the total amount which had diffused through after the period of dialysis indicated in the first column. The rate of dialysis is rapid at first, but quickly diminishes, till after about 21 days it becomes constant at 7 mg. a day, which is equivalent to 0.58 % of the total of the nitrogen originally in the dialyser and 1.6 % of that in the dialyser at this period. These experiments were continued for 60 days without any apparent diminution in the daily rate. After 3 months, however, when the exterior fluid was changed only once every 6 days, a small diminution of the rate of dialysis could be detected. The rate of diffusion is indicated on the curve (Fig. 2). This, it will be observed, becomes practically a straight line after 20 days. These experiments indicate quite clearly the intensely slow rate of diffusion of the degradation products of gelatin, and the impracticability of freeing gelatin from them by any process of washing or dialysis.

The results for the rate of diffusion through parchment of the sample of Coignet's Gold Label gelatin (ash-free) which had been heated for 48 hours at 100° .

Time Days	Quantity of nitrogen diffused in mg.	Total amount of nitrogen diffused in mg.	Percentage of nitrogen diffused through parchment
1	116.0	116.0	9.68
2	68.2	184.2	15.4
3	40.4	224.6	18.6
4	35.5	260.1	21.6
5	29.3	289.4	24.0
6	—	—	—
7	43.1	332.5	27.6
8	22.5	355.0	29.6
9	22.0	377.0	31.4
10	20.3	397.3	33.0
11	18.6	415.9	34.6
12	18.9	434.8	36.2
13	—	—	—
14	24.6	459.4	38.2
15	14.4	473.8	39.5
16	15.0	488.8	40.6
17	9.5	498.3	41.5
18	8.6	506.9	42.2
19	8.5	515.4	42.9
20	—	—	—
21	12.6	528.0	43.8
22	7.8	535.8	44.5
23	7.7	543.5	45.2
24	7.8	551.3	45.9
25	7.8	559.1	46.4
26	7.8	566.9	47.2

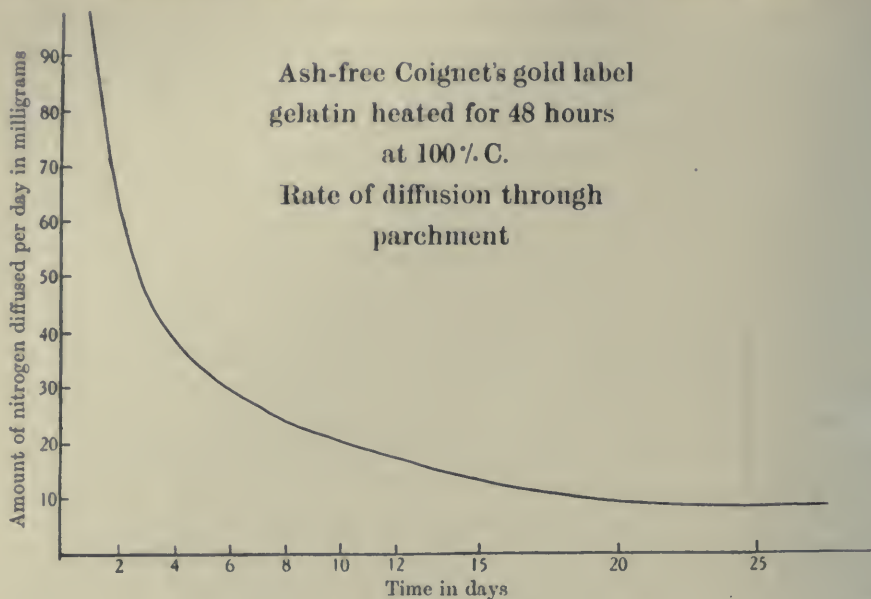


Fig. 2.

A few experiments were carried out with the object of throwing some light on the nature of the thermal degradation of gelatin. No very definite scission of the peptide linkage could be ascertained by either the formalin titration method, or by the estimation of the nitrogen set free by nitrous acid by the method of van Slyke. (There were certain difficulties in applying this method, which will not be discussed in this place.) It was thought that heat might bring about a simple disaggregation of a large molecule into simpler aggregates of the same type. If this were the case, the part of the degraded gelatin which passes the dialyser should yield the same hydrolysis products as the part that remained within. This, however, was not found to be the case as the following determinations of the Hausmann numbers show:

	Amide N	Humin N	Diamino N	Humin + diamino N
Ash-free Coignet's "gold-label" gelatin	1.1	—	22.3	—
A. The same, of which a solution had been heated for 48 hours at 100°	1.2	2.2	21.5	23.5
Dialysis of A through parchment for 21 days:				
(i) Portion which diffused through	1.25	1.3	26.5	27.8
(ii) Portion which did not diffuse	1.1	2.6	20.5	23.1

The diamino-nitrogen of the diffusible nitrogen was distinctly higher than that of the non-diffusible.

SECTION III. EXPERIMENTS ON THE PURIFICATION OF GELATIN BY ELECTROLYSIS.

Loeb's method of purifying gelatin [1922], based on the consideration of its amphoteric character, consists in treating the material first with a solution of hydrochloric acid with a hydrion concentration just greater than that of iso-

electric gelatin and then with water until it is free from acid. It was found, however, that only by very prolonged treatment by this method was it possible to remove the last traces of electrolytes, and it was considered advisable to supplement it by a method of electrolysis. The presence of very small amounts of electrolytes modifies very considerably the properties of gelatin, and an efficient method for obtaining a gelatin which is practically ash-free is, therefore, very essential. It was also necessary to ascertain the efficiency of an electrolytic method for removing the nitrogenous contaminations. The apparatus employed is shown in the accompanying illustration (Fig. 3).

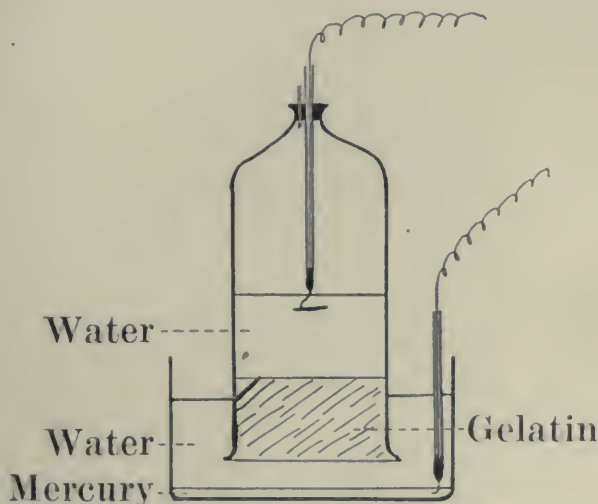


Fig. 3.

One to 1.5 litres of a gelatin solution of 10–20 % were allowed to set in the lower part of a bell-jar of 5 cm. diameter. Both the upper and lower surfaces of the gelatin were in contact with water and a current was passed through the system from a platinum anode to a mercury cathode. The distance from anode to cathode was about 20 cm. The initial voltage employed was usually 100 which was afterwards increased to a maximum of 220. The water was changed once a day at least, and the process was continued until there was no further appearance of alkali at the cathode or of acid at the anode. The gelatin was then removed and cut into slices which were dried in a current of filtered air at ordinary room temperature. The properties of the gelatin thus prepared are referred to in greater detail in the next section.

The gelatin used as the starting material in these experiments was Coignet's "gold-label." It had an ash content of about 1.5 %. The initial washings with dilute acid and water brought this down to 0.4–0.1 %, which was further reduced by the electrolysis to 0.02 % or less. It will be noticed that no membranes were employed for the process. This was found to be possible when the apparatus described above was employed, if the greater part of

the ash content had been first removed. If a sample containing the original amount of ash (1.5 %) was submitted to electrolysis by this method without any previous treatment, it was found that acid gelatin formed towards the anode and alkali gelatin towards the cathode. These underwent swelling, and particles of the jelly escaped into the water, and often the whole jelly broke away from the bell-jar. The method was quite satisfactory, however, for gelatin which had been submitted to a preliminary purification by acid.

Efficiency of the process of electrolysis. A number of experiments were carried out with a view to determine the efficiency of the electrolytic process in removing contaminations other than the inorganic.

The first experiments were made with aniline dyes, and these were carried out in a smaller apparatus than the one described above. This consisted of a simple glass tube of about 5 cm. diameter, which was used instead of a bell-jar; the remainder of the apparatus was practically the same. Into the tube were introduced 50 cc. of a solution of electrolyte-free gelatin (made up from 20 g. gelatin to 100 cc. water), to which the dye was added. The solution was then allowed to set. Methylene blue and methyl red were readily removed from the gelatin by electrolysis, the former to the cathode, the latter to the anode. The colloidal dyes, Congo-red and night-blue were, however, not removed, under the highest potential gradient available (about 25 volts/cm.). Sugars were not removed at all, whilst glycine, an ampholyte with both acid and basic dissociation constants very weak, was only removed very slowly.

Experiments were also carried out to determine the rate of removal of the ordinary nitrogenous contaminants of gelatin. Two 50 cc. portions of an electrolyte-free gelatin (20 g. gelatin + 100 cc. water) were introduced into tubes 5 cm. diameter. One was submitted to electrolysis under a p.d. 60 volts with a distance of 8 cm. between the electrodes. The other tube, used as a control, was not submitted to electrolysis. From time to time the water round the electrodes was removed, and the nitrogen therein estimated. From the results, which are given in the following table, it will be seen that more nitrogenous matter passes out from the electrolysed than from the non-electrolysed gelatin. The experiment indicates the difficulty of complete removal of the nitrogenous contaminants by electrolysis.

		Electrolysed gelatin		Control	
		+	-	+	-
First	4 days	28.3	34.0	4.8	4.6 mg. N
Next	4 "	9.3	11.3	3.0	2.8 "
"	5 "	6.1	6.5	2.4	2.6 "
"	5 "	5.1	5.8	2.0	2.3 "
"	10 "	6.3	6.5	3.5	4.0 "

The electrolyte-free gelatin was found to be extremely resistant to putrefaction.

SECTION IV. THE "RECRYSTALLISATION" OF GELATIN.

A gelatin, which had been purified by the washing and electrolytic processes described above, was found to have properties markedly different from the unpurified. A solution of 2 % or less of the air-dried purified material (which corresponds to about 1.6 % when calculated on the true dry weight) is clear at 40°, but on cooling becomes white and opaque. The gel or solution formed is unstable and on standing for 24–48 hours, the hydrated gelatin separates out in the form of a bulky precipitate, which can be separated from the clear supernatant fluid by centrifuging in the case of the more concentrated solutions or by simple filtration in that of the more dilute solutions. The "recrystallisation" of gelatin, if so it may be termed, can be repeated an indefinite number of times, and results in a further purification of the gelatin.

Solutions of 3 % and higher concentrations set to stable gels on cooling. The 3 % gel is white and opaque, but with increasing concentrations the gels become more and more transparent. The gels of 10 % strength and higher, are, as regards transparency, indistinguishable from those made from ordinary commercial gelatin.

To investigate the recrystallisation process as a means of purification, the nitrogen content of the supernatant fluid obtained by centrifuging and filtering was investigated. The experiments were usually carried out with a litre of solution. After cooling this was generally allowed to stand at room temperature (14–16°) for two or three days, before centrifuging. From a litre of 2 % (air-dried) gelatin, 300 cc. of a clear fluid were usually obtained. After the first "recrystallisation" this contained nitrogen varying from 10–25 mg. per 100 cc. If the separated gelatin is redissolved at 40° and the bulk of the solution is made up to 1 litre, and allowed to cool, the separated clear solution has a nitrogen content of 9–11 mg. per 100 cc. Further "recrystallisations" fail to reduce this amount. Moreover, a solution which had been allowed to stand at room temperature for 6 weeks gave practically the same value for the nitrogen in the supernatant fluid as one which had been allowed to stand for only 48 hours (9.8 and 9.2 mg./100 cc. respectively). This result is in marked contrast with those found for unpurified gelatins.

Another quantity of the "recrystallised" gelatin was washed by decantation with large quantities of water, then separated by centrifuging and again made up to the original volume at 46°. After cooling, the nitrogen content of the supernatant fluid was found to be 10.4 mg./100 cc.

There appears, therefore, to be an equilibrium value of about 10 mg./100 cc. for the nitrogen content of the clear liquid which separates after melting and cooling. This value is not attained unless the solution is heated. When some of the precipitated gelatin was made up to the original volume with cold water without remelting and allowed to stand for 3 days with occasional shaking the supernatant fluid contained only 5.0 mg./100 cc.

When the gelatin is not melted the equilibrium value is only attained

slowly. The following experiments were carried out with a gelatin which had been purified by electrolysis, but not by recrystallisation. 40 g. of gelatin in sheets were allowed to soak in 1 litre of water. After $2\frac{1}{2}$ months, the aqueous phase contained 8.3 mg./100 cc. and after $4\frac{1}{2}$ months 16.2 mg./100 cc. In another case 20 g. gelatin as a 10 % gel was broken up finely and immersed in 800 cc. water. After 2 months the water contained 11.9 mg./100 cc. Experiments on similar lines are being carried out with "recrystallised" gelatin but are not yet completed.

In all the experiments mentioned above, dealing with the supernatant liquid from the "crystallised" gelatin, 2 % solutions of the air-dried material were used. In the following experiments gelatin was allowed to crystallise from solutions of varying strengths. From even the smallest concentrations used the gelatin separates as a hydrate. The amounts of nitrogen in the supernatant liquid are given in the following table.

Initial concentration	N content of clear fluid mg. N per 100 cc.
1.39	11.1
0.697	9.57
0.348	9.38
0.139	11.7

The initial concentrations are here based on the nitrogen determinations in the solutions, it being assumed that pure dry gelatin contains 18 % N. These experiments were not carried out in a thermostat, and the materials used in the determination of the nitrogen contained minute quantities of nitrogenous impurities for which allowance has not been made.

It seems, therefore, that purified gelatin has a real but small solubility in water at the temperatures 14–16° corresponding to about 10 mg./100 cc. N or 0.056 % gelatin.

Unfortunately, further experiment throws some doubt on this explanation of the nature of the apparent equilibrium observed. The filtrate from a solution of the "recrystallised" gelatin was compared with a solution of the gelatin made up directly to the same nitrogen content. On the assumption that we are dealing with a true solubility phenomenon these solutions should be identical. Their viscosity, surface tension and diffusibility through parchment were determined and, within the limits of experimental error, were found to be the same for both solutions.

A litre of each solution was then concentrated under reduced pressure at 40° until the volume was brought to 200 cc. These solutions, which should now correspond to a 0.28 % solution of gelatin, were cooled. *No separation of solid gelatin* occurred from the concentrated filtrate, which was only slightly turbid, whereas from the solution made up directly gelatin separated as from an ordinary 0.28 % solution. This failure to reverse the process and obtain the solid hydrated gelatin from the filtrate on concentration throws doubt not only on the hypothesis of a simple solubility effect but also on the assumption that the results recorded in Section IV above represent an equilibrium. It is

difficult to conceive of any true equilibrium, whether based on solubility, adsorption or disaggregation effects, which would not be reversed by the procedure described. The non-reversibility is not due to non-appearance of the solid phase, since addition of small amounts of the solid does not affect it. In spite then of the agreement of the results of Section IV the existence of a definite equilibrium cannot yet be taken as proved. It is conceivable that gelatin is not a simple molecule but an aggregate. Its molecular weight as determined by Biltz was found to be about 17,000. Repetition of the determination by other methods in this laboratory gave numbers varying between 17,000 and 19,000. It is difficult to conceive that these numbers represent the weight of a simple molecule. The behaviour of such aggregates when dispersed in water cannot yet be predicted.

CONCLUSIONS.

(1) In ordinary high-grade commercial gelatin, the nitrogenous contaminations are not removed even by washing for several months.

(2) The nitrogenous contaminations, which are derived mostly from the thermal decomposition of gelatin during the course of its preparation, contain products which diffuse only very slowly. After several weeks of dialysis of the products obtained from gelatin by diffusion, or of the products obtained by heating purified gelatin in water, in a parchment membrane, diffusible matter is still found to be passing the parchment. The conclusion is drawn that it is practically impossible to free gelatin from its nitrogenous contaminants by washing or dialysis.

(3) It is difficult to free gelatin entirely from electrolytes by washing with acid and water by the method of Loeb. Gelatin purified in this manner, can, however, be readily freed from almost the last traces of stronger electrolytes by submission to electrolysis without the use of membranes. Electrolysis is not, however, efficient in removing substances of colloidal character and non-electrolytes or even weak electrolytes.

(4) Gelatin when purified by washing and electrolysis separates out from solutions of less than 2 % as an insoluble hydrate. When this process of "recrystallisation" is repeated, a gelatin is produced which separates from a supernatant fluid which always contains about 10 mg. per 100 cc. This quantity remains constant, from whatever strength of solution the gelatin separates. When a gelatin with this property is obtained, it indicates that it is free from the soluble nitrogenous contaminants. The gelatin which separates behaves in many respects like a pure substance in equilibrium with a saturated solution, which solution contains 0.056 % gelatin.

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LVIII. SOME NOTES ON THE DETERMINATION OF THE HAUSMANN NUMBERS OF PROTEINS.

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*A Report to the Adhesives Committee of the Department of
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(Received June 6th, 1923.)

THE modification of Hausmann's method by Osborne and Harris [1903] does not give consistent results for gelatin as the following determinations made with three different samples show.

Gelatin	% diamino N
Coignet's Gold label	31.7
	20.8
	20.1
	19.7
Coignet's Gold label which had been washed with frequent changes of water for 10 days	29.9
	29.9
	19.6
Gelatin extracted from ossein at 100°	33.7
	20.3

The percentage of diamino nitrogen varies considerably, and as these results were determined in duplicate under the same conditions and the duplicates were found to give the same result it was concluded that the variations were due to the difference in treatment to which the hydrolysed product was subjected before precipitation.

On referring to the literature it was found that variable results were obtained by different workers.

	% diamino N
Hausmann (1899)	35.83
Van Slyke (1911)	25.5
Bogue (1920)	22.75
	21.68
	22.1
	26.47

These variations are far larger than can be accounted for by differences in the samples analysed and must be due to some faults inherent in the method.

Attempts were therefore made to ascertain the cause of these discrepancies. It was thought that it was due to the formation of a polypeptide under certain conditions. [Compare Dakin, 1920.]

It was quite conceivable that gelatin, in the presence of cold acid, which produces only incomplete hydrolysis, might form certain polypeptides which are only hydrolysed with difficulty.

Therefore experiments were carried out in which the gelatin was heated immediately on adding the acid, and after allowing the gelatin to stand in contact with the cold acid for 24 hours at laboratory temperature.

It was found that the percentage of diamino nitrogen was always higher when the gelatin had been allowed to stand with cold acid for a few hours as the following results show:

	Coignet's Gold label	Gelatin from ossein
Immediately hydrolysed for 12 hours	20.6	25.03
Hydrolysed for 12 hours after standing 24 hours at lab. temp.	24.25	35.62
" " " " 37°	23.7	34.65

It was noticed that where the results were high a secondary precipitate was formed after filtration of the phosphotungstates. This is probably due to the formation of some product of hydrolysis of gelatin which gave a precipitate with phosphotungstic acid which was somewhat soluble in water so that variations in the results were obtained with very slight variations in the conditions.

Experiments were then carried out to determine the rates at which peptide scission and formation of amino groups takes place, with gelatin hydrolysed immediately after mixture with the acid, and after standing with cold acid solutions. It was found that the maximum amount was formed in one hour in the first case and not until after 3 hours in the second case, indicating the formation of resistant groups.

	% hydrolysis	
	A Gelatin hydrolysed immediately	B Gelatin hydrolysed after standing with acid at lab. temp. 24 hours
0 mins.	4.5	8.9
15 "	60.9	—
30 "	62.6	46.6
45 "	68.3	—
60 "	75.8	65.8
1.5 hrs.	75.8	—
2 "	75.8	70.6
3 "	75.4	74.8
6 "	75.7	75.1
28.5 "	75.6	75.0

The rate of hydrolysis was followed by Van Slyke's method for amino nitrogen.

In the case of A the percentage of diamino nitrogen was also determined at various intervals and it was found that although the amino nitrogen reached its maximum after 1 hour the diamino nitrogen did not reach a constant until 12 hours.

Time of hydrolysis in hours	% amino N	% diamino N
3	0.93	36.1*
6	1.0	25.7*
9	1.06	36.6*
12	1.1	20.05
16	1.1	20.09
20	1.1	25.1*†
24	1.1	20.0
28.5	1.23	20.1

* The filtrate from the phosphotungstate precipitate on standing gave a further precipitate.

† This result was abnormal as conditions were not observed which were found out later to have an effect on the results, i.e. the solution was allowed to stand overnight with the acid before precipitating.

It was also found that if the solution containing the diamino acids were allowed to stand some time with 5 % H_2SO_4 abnormal results were obtained.

Gelatin was hydrolysed for 30 hours with 20 % HCl. The amide nitrogen and humin nitrogen were removed. The filtrate was concentrated to 100 cc. and 5 g. H_2SO_4 added. The following results were obtained:

	% diamino N	Type of precipitate
1. Diamino acids precipitated immediately solution was cold	20.4	Granular and easily filtered
2. Diamino acids precipitated after allowing solution to stand 24 hours with 5 % H_2SO_4	23.9	Flocculent ppt. and secondary ppt. which separated after standing
3. Diamino acids precipitated after allowing solution to stand 48 hours with 5 % H_2SO_4	25.1	" "
4. Same as 3 only diamino acids precipitated in warm solution and allowed to stand 24 hours before filtering	20.1	Granular

The results indicate that some change takes place when the amino acids are allowed to stand with acid in the cold. Probably a polypeptide is formed which is precipitated by phosphotungstic acid.

Experiments were carried out to prove the above. The gelatin was hydrolysed for 3 hours with 20 % HCl. After the amide and humin nitrogen had been removed the solution was made up to 100 cc. in 5 % H_2SO_4 and heated for varying lengths of time at 120° in the autoclave. The diamino acids were precipitated with 20 % phosphotungstic acid on cooling.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid after removing amide and humin nitrogen	36.04	Gelatinous
2. Add 3 cc. H_2SO_4 and heat under reflux for 1 hour before precipitating	24.00	Flocculent
3. Add 3 cc. H_2SO_4 and heat in autoclave for 1 hour before precipitating	24.1	"
4. Heat for 2 hours at 120°	23.25	"
5. " 3 " "	20.5	Granular
6. " 4 " "	20.2	"

These results indicate that the correct values for diamino nitrogen may be obtained by hydrolysing immediately for 3 hours, heating the solution with 5 % H_2SO_4 in the autoclave for 3 hours at 120° and precipitating the diamino acids immediately on cooling.

If the solution is allowed to stand a few hours before adding the phosphotungstic acid solution the precipitate will be flocculent or gelatinous instead of granular as the following show:

Gold label gelatin hydrolysed 3 hours with 30 % HCl

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Evaporate to 100 cc. add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid	25.8	Flocculent
2. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours in autoclave at 120°	20.6	Granular
3. As 2 above, but allow solutions to stand 24 hours before precipitation with phosphotungstic acid	30.5	Flocculent with secondary gelatinous ppt.
4. As 2 above; allow to stand 4 days before ppt.	24.1	Flocculent
5. As 4 but filter immediately on adding phosphotungstic acid	20.4	—
6. As 4 above but heat to boiling before ppt.	20.7	Granular

The experiment was repeated with Coignet's "Gold Label Gelatin" which was allowed to stand with the acid 48 hours at laboratory temperature before hydrolysing for 12 hours.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Ordinary way by acidifying solution to 5 % H_2SO_4 and ppt. with 30 cc. of 20 % phosphotungstic acid	24.5	Flocculent
2. Add 3 cc. H_2SO_4 and evaporate to 100 cc. Allow solution to stand 4 days then heat to 100° and when cold add the phosphotungstic acid solution	24.0	„
3. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave and then ppt.	24.5	„
4. Evaporate to 100 cc., add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave. Allow to stand at lab. temp. 2 days before ppt.	30.9	Gelatinous ppt.

The latter results show that when the cold acid is allowed to be in contact with the gelatin for some hours before heating some product is formed which resists hydrolysis even after 12 hours with 20 % HCl and a subsequent heating with 5 % H_2SO_4 at 120° in the autoclave before precipitation of the diamino acids.

In all the above cases where a high value for the percentage of diamino nitrogen was obtained the precipitate formed with phosphotungstic acid was flocculent or gelatinous, and took some time to settle, whereas those which were normal gave a granular precipitate which settled quickly and was easily filtered.

To obtain a correct value for the percentage of diamino nitrogen in the hydrolysis products of gelatin the following precautions should be observed:

1. On mixing the protein and the acid for hydrolysis, the mixture should be heated to boiling almost immediately for 12 hours.

2. The solution which contains the diamino acids should not be allowed to stand any length of time before the phosphotungstic acid solution is added. If the solution has been standing with the H_2SO_4 it should be heated to boiling for a few minutes and when cool the diamino acids precipitated with 20 % phosphotungstic acid solution.

A correct result for the percentage of diamino nitrogen may be obtained by hydrolysing the gelatin for 3 hours with 20 % HCl at boiling point and, after removing the amide and humin nitrogen, adjusting the solution containing the diamino acids to 200 cc. and adding 3 cc. H_2SO_4 . This solution should then be heated for 3 hours in the autoclave at 120° and when cool the phosphotungstic acid solution should be added. This method cannot be applied when the complete analysis is required seeing that the amide group is not entirely broken down until the gelatin has been hydrolysed with 20 % HCl for 12 hours.

All the difficulties encountered hitherto, appear to be due to the fact that the hydrolysis products condense in the presence of cold acids and these condensation products cause the precipitation of gelatinous phosphotungstates.

Experiments were made to find another precipitant for the diamino acids other than phosphotungstic acid. The following alkaloidal reagents were tried: Bruckes' reagent, Mayer's reagent, cadmium iodide, tannic acid. None of these gave a precipitate with diamino acids in acid solution. Tannic acid only precipitates in neutral solution. This precipitate was examined and found to give the same result as the phosphotungstic acid precipitate.

The above work was carried out under the direction of Professor S. B. Schryver.

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LIX. THE HEMICELLULOSES. I. THE HEMICELLULOSE OF WHEAT FLOUR.

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SOME years ago, Schulze described the preparation of nitrogen-free residues from leguminous seeds, which yielded on hydrolysis with dilute acids reducing sugars. The precursors of these sugars which were not actually isolated were called by Schulze "hemicelluloses¹."

During the course of some researches on the constituents of the cell-wall substances of plants by Clayson, Norris and Schryver [1921], it was found that, by means of *N* sodium hydroxide solution, substances could be extracted from all the materials investigated, which were soluble in the more concentrated alkaline solutions, but not in very weak alkali; they could be precipitated from the alkaline solutions on addition of acids, and as they yielded reducing sugars on hydrolysis with acids, they were assumed to belong to the class of products termed by Schulze the "hemicelluloses." Most of the products isolated gave on hydrolysis relatively large amounts of pentoses, and all of them gave a blue coloration with iodine.

It was decided to continue investigations with the object of isolating in bulk these so-called "hemicelluloses" and of determining their distribution and later on their function in plant-tissues. The distribution appears to be a very wide one. In some lignified tissues they exist in relatively large quantities ("wood-gums"), and the product isolated many years ago from barley by O'Sullivan and called by him "amylan" also apparently belongs to this class.

The first investigations were carried out on leguminous seeds from which a "hemicellulose" and also "cytopeptic acid" [see Clayson, Norris and Schryver, 1921] were isolated and in extending the work to cereals wheat flour was chosen as the chief material for research. By means of the process described in detail below, a product giving a blue coloration with iodine like starch, but differing in many essential properties from this substance, was isolated. It is shown in the second communication [Schryver and Thomas, 1923] that the same product can also be isolated from the commercial starches.

¹ A fuller account of the literature of the hemicelluloses is given in the third paper of this series by Miss O'Dwyer [1923].

EXPERIMENTAL.

Extraction of gliadin. Flour in quantities of 1 kilo. at a time was heated in tin-lined copper vessels, provided with a reflux condenser, with 8 litres of 70 % alcohol. An efficient stirrer, mechanically driven, was attached to the apparatus and the whole mass kept stirred whilst the mixture of flour and alcohol was heated in a water-bath. After about two hours the alcohol was filtered off from the flour on a Buchner funnel, and the flour was returned to the extraction apparatus and treated a second time with 70 % alcohol. By this means nearly all the gliadin was extracted from the flour. This, after the second filtration from the alcohol, was air-dried.

Extraction of the starch. The dried gliadin-free flour was then ground up to a thick paste with water by a motor-driven pestle and mortar and the paste was then thrown into nearly boiling water. About 9 litres of water altogether were used for each kilo. of flour. The mixture of flour and water (which was made in enamelled buckets) was then kept for 1 hour at about 85° by immersing the containing vessel in a bath of boiling water and was stirred from time to time. In this way the starch was gelatinised. The mass was then cooled to 40° and 1 g. of taka-diastase, dissolved in about 500 cc. of water at 40° was added gradually with constant stirring. Toluene and chloroform were then added and the mixture was kept for about 20 hours in an incubator at 37°. After this period, the mixture had lost its gelatinous consistency, and was filtered through coarse muslin, with hand pressure, to separate the undigested lumps. These were afterwards ground up with water in a motor-driven pestle and mortar, and the paste thus obtained either added to the main bulk of the filtrate or to the second digestion mixture obtained by the method about to be described. The filtrate was then passed two or three times through a Sharples centrifuge, using first a wide, and afterwards a finer nozzle, the centrifuging process being repeated (generally three times) until the liquid was nearly clear. The solid matter was then obtained in the form of a thick paste. This was then ground up with water, and, as before, the paste was thrown into nearly boiling water, 9 litres again being used. The mixture was kept at about 80° for 1 hour and again digested with taka-diastase. After the second digestion, the mass usually passed through coarse muslin without pressure and contained very few lumps. The solid was again separated from the liquid by the Sharples centrifuge, and gelatinised and redigested with taka-diastase as before, for a third time. In the third digestion, only about half the bulk of water used in the first digestions was employed. In the earlier experiments the course of digestion was followed out by estimations of sugar. It was found that by far the greater part of the starch was digested on the first treatment with taka-diastase. Practically no sugar was obtained as the result of the third digestion, but this was always carried out as a precautionary measure. The starch-free product was obtained finally in the form of a thick paste.

Extraction of the glutenin. The glutenin is soluble in 0.1 % sodium hydroxide

solution, which is too weak to extract the hemicellulose. In some experiments, the starch-free product obtained as above was dried in a current of air before the extraction of the glutenin. It was then obtained in the form of a very hard horny mass, from which the glutenin is extracted only with very great difficulty. It is therefore advisable to extract the glutenin from the starch-free paste without drying previously. This is accomplished by repeated extractions with weak alkali, the solid being afterwards separated from the liquid by means of the Sharples centrifuge. The extraction should be continued until the liquid no longer gives the biuret reaction. It is difficult to get out the last traces of nitrogen, but these can be eliminated by methods of purification described below.

Extraction of the hemicellulose. The mass remaining after extraction of the glutenin is then treated with cold 4 % solution of sodium hydroxide. An appreciable amount of yellowish amorphous substance remains undissolved. This contains a small amount of cytopeptic acid, which could be extracted from it by 0.5 % ammonium oxalate solution by the method previously described [Clayson, Norris and Schryver, 1921]. The remainder consisted of cellulose, which dissolved completely in zinc-chloride-hydrochloric acid solution. The insoluble mass was separated from the liquid by filtration through a thick pad of paper pulp (made up with 4 % caustic soda solution). The filtrate was slightly opalescent. On acidification with acetic acid, it became at first very turbid, but on standing for a short time a very bulky white precipitate separated, leaving a perfectly clear white supernatant fluid. This was filtered off on to a large filter, thoroughly washed with water, graded strengths of alcohol, and then with ether and finally air-dried. It was thus obtained in the form of a light white powder.

Properties of the hemicellulose. When prepared in the manner just described, the flour hemicellulose dissolves fairly readily in boiling water, giving a perfectly limpid solution, from which the hemicellulose very readily separates again on cooling. The solution can be filtered through paper in a hot water funnel. The hemicellulose on drying at 100° loses about 50 % of its weight, and the apparently dry powder holds large quantities, therefore, of the liquids which have been used in its preparation. After drying at 100° it is obtained in the form of a hard granular powder, or sometimes of a horny mass. When in this condition, it does not dissolve readily in water boiling under atmospheric pressure. It readily dissolves, however, when heated with water in an autoclave to 120°. From this solution it readily separates in a non-crystalline form on cooling. It gives a blue coloration with iodine just as ordinary starch does, but resists digestion with taka-diastase. It does not reduce Fehling's solution; when boiled with 1 % sulphuric acid it undergoes hydrolysis, yielding glucose (see next paper). This hydrolysis takes place rapidly and is nearly complete after 2 or 3 hours. As usually prepared, it contains small quantities of nitrogen. It can be purified either by allowing it to separate from its filtered solution in hot water, or by means of the copper salt, a method which is described in detail in the following paper. By these methods it can

be obtained quite free from nitrogen and ash, and then shows a rotation of $[\alpha]_D = +150^\circ$. It is in all respects identical with the hemicellulose obtained from starches, which are described in the paper just quoted.

Origin of the hemicellulose. The hemicellulose was first prepared from flours, purchased in shops, of which the origin was quite unknown. Very varying yields were obtained from different flours. It was of interest to ascertain what the origin of this "hemicellulose" was. It was conceivable that it was an intermediate product derived by the digestion of ordinary starch by taka-diastase. On the other hand, it might be a distinct entity derived either from the starch grains, or from a different part of the plant cell (possibly the cell-wall). To test these points, an estimation of the cellulose was made from two flours of known origin. These were kindly supplied by Dr A. E. Humpharies. They were both obtained from the same mixture of Australian, English and Manitoba wheat. One flour was, however, a "straight-run" flour corresponding to a yield of 73 % of the wheat, whereas the other was more refined and corresponded only to a 23 % yield. It was found that the former gave a yield of 5.4 % hemicellulose (calculated as dried at 100° and protein-free) whereas the latter gave a yield of only 3.6 %. These experiments were each carried out with 1 kilo. of flour. The hemicellulose obtained from each sample (by the methods described above) was not completely purified, but the nitrogen and dry weight were estimated in each. These preliminary experiments on which not too much weight can be put, indicate that the hemicellulose is not derived from the endosperm.

It is possible that the hemicellulose in flour may exert a considerable influence on its value for bread-making and it is proposed to investigate this question in detail.

The authors wish to express their thanks to Dr Humpharies for his assistance in providing materials and one of them is indebted to the department of Scientific and Industrial Research for a grant which enabled him to carry out this work.

SUMMARY.

From wheat flour can be isolated a substance which resists the action of taka-diastase, and is soluble in hot water, in which it gives a perfectly limpid solution from which it separates readily on cooling in an amorphous form. It is soluble in N NaOH, from which solution it can be precipitated by acids. It can be purified either by separation from its solution in hot water, or by means of its copper salt, and when thus obtained, nitrogen- and ash-free, has a rotation in $N/2$ NaOH of $[\alpha]_D^{20} = +150^\circ$.

(Note. A fuller description of some of the properties is given in the following paper.)

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LX. THE HEMICELLULOSES. II. THE HEMICELLULOSE CONTENT OF STARCHES.

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IN the first paper [Clayson and Schryver, 1923] a "hemicellulose" very similar to starch but differing from this in many particulars has been described. Some evidence has been given to show this is not an intermediate product of degradation of ordinary starch by taka-diastrase, but a distinct chemical entity. If this were the case, it might be expected that it would be yielded in different amounts by the digestion of ordinary starches and that there might exist some correlation between the amount obtained and the properties of the various starches investigated. This has been found to be the case, and yields varying between practically 0 and 4 % have been found in the six commercial varieties of starch which have been studied.

Preparation of the hemicellulose from starches.

The six starches investigated were those from sago, maize, wheat, rice, tapioca and potato. The authors are indebted to Messrs Hibbert and Co. of Chorley, the Corn Products Co., Manchester, and Young and Strang of Glasgow for their kindness in providing materials for the research.

The method of preparation of the hemicellulose was practically identical with that employed for obtaining it from gliadin-free wheat flour as described in the first paper. The material was gelatinised and treated three times with taka-diastrase, the filtrations after each digestion being carried out by means of the Sharples centrifuge in exactly the manner therein described. The residue after digestion and treatment with 0.1 % caustic soda was dissolved in 2-4 % caustic soda solution, from which the hemicellulose was precipitated by acetic acid.

With potato starch, which gives a nearly clear gel on gelatinisation, only an insignificant residue was obtained after two digestions with taka-diastrase, whereas sago and maize starches, which yielded on gelatinisation thick pastes,

gave relatively large yields. In each case the digestion was carried out with 1 kilo. of starch and the following yields were obtained:

Origin of starch	Crude hemicellulose %	Nitrogen content %	Nett yield of hemicellulose
Sago	4.1	1.3	3.8
Maize	3.9	1.35	3.6
Tapioca	2.0	1.7	1.7
Wheat	1.65	2.1	1.35
Rice	1.2	2.3	1.0
Potato	Practically none	—	—

The hemicellulose still contained nitrogen (column 3), and if this be in the form of protein, the nett yields of hemicellulose from the various starches may be calculated as given in the last column.

Purification of the crude hemicellulose.

Washing the crude hemicellulose with 0.1 % caustic soda solution was not found to be completely effective in removing the protein. Separation of the hemicellulose from a filtered solution in hot water was somewhat difficult, owing to the ease with which deposition took place, even when a hot-water funnel was used. A method similar to that employed by Baker and Pope [1900] was used. The hemicellulose was dissolved in 2 % NaOH solution and copper sulphate was added till no further precipitation took place. The light blue precipitate was centrifuged off, and washed repeatedly on the centrifuge with 0.2 % NaOH solution. It was then treated with dilute acetic acid and the product thus obtained was washed until the blue copper coloration was removed. It was then treated with graded strengths of alcohol, and then ether and finally air-dried. The white flocculent mass thus obtained still held large amounts of the liquids used in the preparation, but could be readily dried to constant weight by heating to 110°, when it was obtained in the form of a granular powder, free from nitrogen and ash. The specific rotations of the samples in $N/2$ NaOH obtained from the different varieties of starch were practically identical, viz.:

Sago	+ 151.6°
Maize	+ 150.6
Wheat starch	+ 150.0
Tapioca	+ 151.0
Rice	+ 150.9
Wheat flour	+ 150.6

The product gave the following results on combustion:

I. C. 41.3 %; H 6.8 %. II. C 41.6 %; H 6.7 %.

This corresponds with a formula $C_{18}H_{34}O_{17}$ or $(3C_6H_{10}O_5 + 2H_2O)$ (this latter is intended only as an empirical representation of the formula) which requires C = 41.3 % and H = 6.5 %.

Hydrolysis of the hemicellulose.

The hemicellulose is readily hydrolysed by dilute acids. About 20 g. of material were purified by the copper salt method, and the purified product,

without drying, was put immediately into a flask containing about 300 cc. of 1 % sulphuric acid and heated with a reflux condenser for about 6 hours. During the first hour, the greater part of the product disappeared, but the solution remained turbid even after quite prolonged heating. In this and every other hydrolysis carried out, there was always obtained a small amount of a white flocculent substance, which obstinately resisted hydrolysis. The nature of this material, which is only formed in small quantity has not yet been ascertained. After its removal by centrifuging, the clear liquid was treated with the exact requisite amount of barium hydroxide, the barium sulphate was filtered off, and the filtrate concentrated to a syrup under diminished pressure. The syrup on treatment with absolute alcohol gave an almost white solid. From a solution of this an osazone was obtained in large yield, which under the microscope appeared to consist entirely of glucosazone, uncontaminated by the osazone of any other sugar. After recrystallisation it gave a m.p. of 204° . A diphenylhydrazone was also prepared, which agreed in properties with the diphenylhydrazone of dextrose. All attempts to discover other sugars (Seliwanoff's reaction for fructose, Tollens' pentose reaction, etc.) yielded negative results. The solution did not, however, contain pure glucose, for quantitative estimations by Bertrand's method and rotation determinations carried out on the same solution gave instead of a specific rotation of about $+52^{\circ}$, one of about 30° . The glucose was, therefore, contaminated with a substance of lower or opposite rotation.

In other samples of the solution an effort was made to estimate the glucose, by determining the total solids and their reducing power by Bertrand's method. The results of two experiments were as follows:

	I	II
Total solids present	0.1130	0.1445
Glucose therein by Bertrand's method	0.1200	0.1469

The reducing power is therefore a little larger than it should have been if the total solids had consisted entirely of glucose.

Finally, an effort was made to obtain the glucose pure by a method employed by Monier-Williams [1921]. The syrup obtained by the hydrolysis of hemicellulose was taken up in pure methyl alcohol. A small amount of amorphous matter remained undissolved. The filtrate from this was evaporated to a syrup which was again taken up with methyl alcohol, the solution was boiled with animal charcoal, filtered, and evaporated to a syrup. This on standing in a desiccator commenced to crystallise. A small portion of it was dried at 100° and the rotation of the solid residue was determined. This was found to be about $+40^{\circ}$. The crystals which separated out had an $[\alpha]_D^{20}$ of $+52.4^{\circ}$. There is no doubt, therefore, that the main product of hydrolysis is glucose. This is, however, contaminated with a small amount of other substances including one insoluble in water. Probably they are of a dextrin-like character, such as are generally obtained on the hydrolysis of starch by dilute acids. On hydrolysing the hemicellulose with 1 % oxalic acid at 120° an insoluble product was also obtained.

SUMMARY.

A method is described for isolating from the starches a hemicellulose identical with that previously described as having been obtained from wheat flour. The substance has an $[\alpha]_D$ in $N/2$ sodium hydroxide solution of $+150^\circ$. It has a composition corresponding with $C_{18}H_{34}O_{17}(3C_6H_{10}O_5 + 2H_2O)$. It yields on hydrolysis with dilute acids glucose as the only reducing sugar mixed with small amounts of other products, probably of a dextrin-like character. The various starches give yields of this substance varying from practically 0 in the case of potato starch up to nearly 4 % in the case of sago.

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LXI. THE HEMICELLULOSES. III. THE HEMICELLULOSE OF AMERICAN WHITE OAK.

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Introduction. The term "hemicellulose" was employed by E. Schulze [1892] and B. Schulze [1894] to denote a substance somewhat similar in character to cellulose, which is easily hydrolysed by weak acids.

E. Schulze [1892] isolated this substance from leguminous seeds by treatment with dilute alkali, and then precipitating the filtrate obtained with dilute acid. He also obtained it from wheat bran and rye bran by the same method. On hydrolysing the product obtained from all three sources, he found that it yielded, in each instance, arabinose and xylose.

Schulze and Tollens [1892] obtained xylose from wheat straw and maize stalks by the same method. From beech wood sawdust Wheeler and Tollens [1889] obtained xylose, and Allen and Tollens [1890] found the same sugar in cherry wood.

Ulander and Tollens [1906] obtained from the hemicellulose of lichens on hydrolysis, glucose, mannose and galactose; while Tottingham, Roberts and Lepkowsky [1920] found xylose, glucose and small amounts of galactose in the alcohol-soluble fraction of the products from the acid hydrolysis of the hemicellulose of apple wood.

Castoro [1906] found that hemicellulose from the seeds of *Ruscus aculeatus* yielded mannose and a small quantity of arabinose on hydrolysis. From the husks of the same seed he obtained galactose.

Again Castoro [1907] found that the husks of *Cucurbita Pepo* seeds contained xylan, as xylose crystals were isolated from the syrup obtained after the husks had been hydrolysed with sulphuric acid, and that a galactan was also present, as the mother liquor from the xylose crystals yielded mucic acid when oxidised.

E. Schulze and Pfenninger [1910] obtained some interesting results from the pods of *Pisum sativum* and of *Phaseolus vulgaris*. Both of these were found to be rich in hemicellulose, the amount increasing as maturation advanced. Thus the unripe pods of *Pisum* gave 16–19 %, whilst the ripe pods yielded 48.6 %. On hydrolysis, galactose and arabinose were obtained, but the hemicellulose was not actually isolated.

Although isolated instances of this kind occur throughout the literature,

no systematic attempt seems to have been made, so far, to examine the "hemicelluloses" obtained from various sources, and to compare the results obtained in each case. Most of the estimations seem to have been carried out on seeds.

Before the work described in this paper was begun experiments carried out in this laboratory by Miss E. M. Thomas [Schryver, 1922] on small amounts of American White Oak sawdust, showed that alkali extracted substances in amounts varying greatly in different specimens of timber. These substances appeared to be somewhat similar to those obtained by E. Schulze [1892], and called by him the "hemicelluloses."

It was then decided to endeavour to isolate the product, or products, from large quantities of the American White Oak, and to investigate its properties and the products of hydrolysis. The sawdust employed was obtained directly from the cooerage of the brewery of Messrs Whitbread and Co., through the courtesy of Mr H. C. Sweetman. No further information as to its origin was available beyond the fact that it was from American White Oak. It is hoped later to continue the investigation of the hemicelluloses from timbers, the origins of which are authenticated, with the object of determining the distribution and functions of these substances.

Method of Preparation. In the first instance, 600 g. of the sawdust were extracted several times with hot water and dried. 6 litres of 4 % caustic soda were then added to the dried material and the mixture was left standing for two days, during which time it was well stirred with a mechanical stirrer. It was next filtered through fine muslin on a Buchner funnel, the sawdust being well washed with weak alkali. A slight excess of acetic acid was added to the filtrate and washings, and a greyish-white precipitate of a sticky character which was found to be partly re-distributed in water, was formed. The filtrate appeared to be of a colloidal nature and precipitation was not complete until an equal volume of 95 % alcohol had been added. The precipitate brought down by the alcohol was a flocculent one. The whole of the precipitate was then collected on a filter, washed with 50 % alcohol, and purified by re-dissolving in 4 % caustic soda, filtering through paper pulp, and re-precipitating with glacial acetic acid, and 95 % alcohol. After washing with various grades of alcohol and finally with ether, the resulting product was dried first in the air, and then in the oven at 105° and weighed. It was found that 62.45 g. of the products were obtained from 600 g. of sawdust, a yield of a little over 10 %.

50 g. of this material were now further purified by a method described by Baker and Pope [1900]. It was first extracted with 2 % caustic soda on the water-bath, and filtered through glass wool. An excess of Fehling's solution was then added, and the voluminous blue precipitate was washed several times with very dilute alkali. Cold dilute acetic acid was then added to decompose the copper compound, and the mixture washed first with very dilute acid and then with dilute alcohol. The precipitate was next treated with boiling absolute alcohol and washed with cold 95 % alcohol to get rid of the

last traces of acid. After washing with alcohol and then with ether, it was allowed to dry, first in the air, and then in the oven at 105°.

Properties. The product obtained by this method consists of a fine greyish-white amorphous mass, free from nitrogen and from inorganic matter. It is soluble in boiling water, forming a thin gelatinous mass on cooling. It does not dialyse through parchment to any extent. On addition of iodine to the solution, a slightly greenish-blue coloration is given. It does not reduce Fehling's solution and is laevo-rotatory. After purification of the product by the copper method as described above, the rotation $[\alpha]_D = -75^\circ$, in 1 % caustic soda solution, was found. The following figures were obtained by combustion:

(1) 0.1874 g.; 0.3070 CO₂; 0.1089 H₂O; C = 44.67 %, H = 6.40 %.

(2) 0.1974 g.; 0.3239 CO₂; 0.1130 H₂O; C = 44.74 %, H = 6.35 %.

The examination of the hydrolysis products shows that the substance is probably a mixture of nearly 70 % xylan + araban, and 30 % mannan + galactan, a very small percentage of material remaining undissolved after hydrolysis. A mixture of 70 % araban + xylan (C₅H₈O₄) and 30 % mannan + galactan (C₆H₁₀O₅) requires C = 45.14 % and H = 6.12 %.

On hydrolysing 0.4 g. of the product with 12 % hydrochloric acid and treating the distillate with phloroglucinol, the amount of furfural phloroglucide precipitated was found to be 0.2730 g., which, reckoned as pentose, gives a yield of 70 %. On treating the contents of the crucible containing the furfural phloroglucide with alcohol, according to the method described by Tollens and Ellett [1905], for the identification of methylpentose, no loss in weight occurred, and the alcoholic filtrate showed no change in colour. It is indicated therefore that there is no methylpentose present.

In order to ascertain the relation between the time of hydrolysis of the product with dilute sulphuric acid and the amount of reducing sugars present, 0.5 g. of the product was subjected to hydrolysis with 1 % sulphuric acid for periods of two, three, four and six hours. Bertrand's method was employed to ascertain the reducing power of the hydrolysed solution in each case.

It was found that:

After 2 hours' hydrolysis 43.74 %			
„	3	„	51.24 „
„	4	„	59.0 „
„	6	„	61.74 „

of reducing sugar, reckoned as glucose was obtained.

THE ISOLATION OF THE HYDROLYSIS PRODUCTS.

Preliminary Experiments. These preliminary experiments were carried out with a product which had not been purified by the copper method.

20 g. of the product were hydrolysed with 200 cc. of 1 % sulphuric acid for six hours. The hydrolysed solution at the end of this time still contained a small proportion of insoluble matter. The solution was filtered and a suffi-

cient quantity of barium hydroxide added exactly to neutralise the solution, which was then filtered and concentrated to small bulk under reduced pressure. The solution was now in a syrupy condition. Some 95 % alcohol was added to get rid of any barium sulphate remaining, and after filtering, the solution was again concentrated as before. A little more alcohol was then added and the syrup was allowed to evaporate slowly over sulphuric acid. The crystalline mass which slowly formed was then heated for a long time on the water-bath with 95 % alcohol [E. Schulze, 1892] and left to stand over concentrated sulphuric acid. After some time a white crystalline mass formed. The mother liquor was then filtered off and to 20 cc. of this, 2 g. phenylhydrazine hydrochloride and 3 g. of sodium acetate were added. The mixture was heated in a test-tube in a boiling water-bath for ten minutes and at the end of that time an orange-yellow osazone separated out. This was filtered off, and on again heating the mixture in the water-bath for some time indications of a second osazone appeared, with the formation of some brownish oily drops.

The first formed osazone was now washed with water and dried and gave a melting point of 156° . On recrystallisation from 60 % alcohol the constant melting point was found to be 160° . It appeared to be the osazone of xylose. As a means of further identification Bertrand's reaction [1891] was now carried out on another portion of the solution from which the osazone was formed. To 2 g. of the sugar syrup, 2.5 g. of bromine and 5 g. of cadmium carbonate were added. The mixture was then gently warmed in a test-tube and after corking loosely set aside for twenty-four hours. At the end of that time the solution was evaporated almost to dryness, taken up with water and again evaporated almost to dryness. On the addition of a little alcohol a crystalline mass formed which at first appeared somewhat amorphous under the microscope, but on recrystallisation from alcohol, the characteristic boat-shaped needles of the double cadmium salt of xylose $(C_5H_9O_6)_2 Cd + Cd Br_2 + 2H_2O$ were deposited. A third portion of the same sugar syrup was boiled with some paraformaldehyde and after a time a white mass of crystals separated out. These were found to have a melting point of 55° . *l*-Xylose diformal melts at 56° and has the formula $C_5H_6O_5(CH_2)_2$ [Lobry de Bruyn and Van Eckenstein, 1903]. The osazone of *l*-xylose, the double cadmium salt of the same sugar, and *l*-xylose diformal have therefore been obtained from different portions of the same sugar syrup.

The mass of crystals which had been filtered off from the mother liquor were now dried and examined. 1 g. was treated as before with 2 g. of phenylhydrazine hydrochloride, 3 g. of sodium acetate and 20 cc. of water [Mulliken, 1905] and after heating in a test-tube in a boiling water-bath for a very short time some colourless crystals separated out. After more than fifteen minutes an orange-yellow osazone formed and brownish-yellow oily drops appeared at the surface. After filtering off the osazone, washing as before, and recrystallising from 60 % alcohol, it was found to have a melting point of 160° . As the osazones of both xylose and arabinose give this melting point, further

means of identification had to be sought. An attempt was therefore made to obtain the diphenylhydrazone of arabinose by treating the syrupy solution of the sugar with slight excess of diphenylhydrazine [Neuberg, 1900, 1904; Browne, 1912]. Sufficient alcohol was added to form a perfectly clear solution and the mixture was boiled for half an hour in a water-bath in a flask connected with a reflux condenser. After standing for some time a white crystalline mass separated out from the solution. The crystals were washed with a very little cold alcohol and dried in the water oven. They gave a m.p. of 196° . After recrystallisation a m.p. of 199° was obtained with rapid heating. Tollens and Maurenbrecher [1905] observed a m.p. of $204^{\circ}-5^{\circ}$ for the diphenylhydrazone of arabinose, but Haar [1920] points out that it is difficult to obtain a constant m.p. higher than 200° .

Second Hydrolysis. 1200 g. of sawdust were now subjected to the same treatment as before, and yielded 121.05 g. of the product; 60 g. of which were hydrolysed with 300 cc. of 1 % sulphuric acid. The hydrolysed solution was again neutralised with barium hydroxide and concentrated as before. Four volumes of 95 % alcohol were then added and the solution, after filtering, was concentrated a second time to very small bulk and decolorised with norite. After further addition of alcohol and a third concentration, the resulting syrup was divided up into two portions. Absolute alcohol was added to the first portion and a large amount of precipitate formed. This, after drying, was treated with hot 80 % alcohol and filtered. As mannose is soluble in hot 80 % alcohol it was hoped to obtain the greater part of this sugar, if present, in the filtrate. This filtrate was again divided into two portions, one of which was left standing over concentrated sulphuric acid. To part (a) of the remainder, one volume of phenylhydrazine and one volume of 50 % acetic acid were added. After the mixture had stood for a short time, colourless rhombic crystals were formed. These were left in the solution over the week-end and had disappeared on the following Monday morning. On warming the solution for a short time a mass of sheaf-like crystals, entirely different from those first formed, appeared. They closely resembled crystals of glucosazone under the microscope. After recrystallisation from 60 % alcohol they gave a constant melting point of 204° . This melting point is that of the osazone of mannose.

Part (b), the remainder of the second portion of the last filtrate, was treated with phenylhydrazine and 50 % acetic acid as before. After standing for a short time the rhombic crystals again appeared. Some colourless needles were also seen under the microscope. The crystal mass was collected after twenty-four hours, dried and recrystallised from hot water. The melting point before recrystallisation was found to be 178° with rapid heating; after repeated recrystallisations from hot water a fairly pure hydrazone was obtained, melting at 196° . The low melting point obtained in the first instance is probably due to the fact that some crystals of the hydrazone of galactose were present. $[\alpha]_D$ (in pyridine) = $+22^{\circ}$. Haar [1920] gives a value for mannose phenylhydrazone $[\alpha]_D = +26.61^{\circ}$.

That portion of the solution obtained with hot 80 % alcohol which had been left standing over sulphuric acid, was now examined. Part of it was found to have separated out as a hard white amorphous mass. On drying this and treating with 90 % alcohol under reduced pressure crystals were formed after the syrup had stood a short time. These were dried on a suction filter and washed with small amounts of alcohol and ether, and then recrystallised from alcohol. They gave a constant melting point of 136° . $[\alpha]_D$ (in 10 % aqueous solution) = $+15.3^{\circ}$. On adding phloroglucinol to the sugar solution no coloration was given. The crystals are evidently mostly those of mannose, with probably a little galactose also present. The rest of the syrup obtained from the second hydrolysis with 1 % sulphuric acid was used later on for a rough qualitative estimation of two of the sugars which had previously been identified.

Third Hydrolysis. 20 g. of the purified product were hydrolysed as before. There was still present a small insoluble residue which after drying and weighing, was found only to amount to 0.45 %. The hydrolysed solution was neutralised as before with barium hydroxide, treated with alcohol and evaporated to a syrup. After concentrating and taking up with alcohol three times an attempt was made to separate the sugars, which previous experiments had shown to be present, by their differences in solubility in alcohol and in water. The results have been fairly satisfactory and it is hoped that further work along these lines may be even more successful. The sugar syrup was decolorised with norite, and, after concentration the third time to very small bulk under reduced pressure, was dried over sulphuric acid, precipitated with hot absolute alcohol and filtered immediately. As xylose is soluble in hot absolute alcohol to a much greater extent than mannose, arabinose or galactose, it was hoped to obtain this sugar in a fairly pure condition in the filtrate. The filtrate was again concentrated under reduced pressure and left to crystallise out over sulphuric acid. After some time crystal aggregates consisting for the most part of fine needles appeared. These were sucked dry on the filter, washed with a little alcohol and ether and then recrystallised from alcohol. They gave a melting point of 156° . $[\alpha]_D$ (in 10 % aqueous solution) = $+20^{\circ}$. On heating a solution of the sugar with phloroglucinol, a greenish-black precipitate appeared. According to Mulliken [1905] the melting point of xylose is $150-153^{\circ}$ and its specific rotation $+18.7^{\circ}$.

The precipitate obtained by adding hot absolute alcohol was left dry over sulphuric acid and then just sufficient water was added to dissolve up the crystals. After concentrating to a slight extent under reduced pressure, the solution was left over sulphuric acid. Crystals began to separate out from the aqueous solution. These were collected and dried on the suction filter, the mother liquor being again treated with absolute alcohol and left over sulphuric acid. The crystals, on drying, gave a melting point of 116° . On recrystallisation from absolute alcohol a mass of fine hexagonal crystals formed. These melted at 167° . A solution of the crystals gave a very slight coloration with phloro-

glucinol. Galactose [Mulliken, 1905] melts with rapid heating at 168° and has a specific rotation of $+80.3^{\circ}$. A value for $[\alpha]_D$ of $+82^{\circ}$ was obtained in 5 % aqueous solution. Owing to the small amount of the sugar present, the mucic acid estimation [Fernau, 1909] could not be carried out at this stage, but it was done later on a solution containing a mixture of the sugars. The phenylhydrazone was obtained by dissolving some of the sugar crystals in a little water and adding phenylhydrazine and 50 % acetic acid as before. After the mixture had stood for about two hours the hydrazone separated out in the form of fine colourless needles, similar to those seen when the hydrazone of mannose was prepared. These were washed with ether and then with a little alcohol and ether. After drying, these were found to have a melting point of 156° . The specific rotation was not obtained. The mother liquor from the last filtration which, it was thought, would probably contain arabinose, some mannose, and perhaps a little galactose was concentrated under reduced pressure and treated several times with hot absolute alcohol. Mannose is slightly soluble in hot absolute alcohol, and galactose is a little less insoluble in the same reagent than arabinose, hence after repeated extractions with hot absolute alcohol, it was hoped to obtain a fairly pure specimen of arabinose. The precipitate which remained after these extractions was dried on the suction filter after standing over sulphuric acid and recrystallised from hot absolute alcohol. The crystals were filtered off, washed with a little alcohol and ether and dried over sulphuric acid. They gave a constant melting point of 160° . $[\alpha]_D$ (in 10 % aqueous solution) = $+103^{\circ}$. On treatment of the sugar solution with phloroglucinol a purplish-black precipitate appeared.

The sugar is therefore identified as *l*-arabinose.

In order to obtain an approximate idea of the amounts of xylose and arabinose present, the diphenylhydrazone of arabinose was obtained by the method described in a previous part of this paper. The mixed sugar syrup from the second hydrolysis, after being treated with alcohol, and concentrated under reduced pressure was allowed to crystallise out over sulphuric acid. 2 g. of the mixed sugar crystals were then dissolved in a little water and 2.5 g. diphenylhydrazine added. When the hydrazone had separated out, the crystals were filtered into a weighed Gooch crucible, washed with a very little alcohol, dried in the water oven, and weighed. It was found that 0.780 g. of diphenylhydrazone $C_{17}H_{20}O_4N_2$ was obtained from 2 g. of the mixed sugars. In order to obtain the amount of arabinose ($C_5H_{10}O_5$) which this figure represents, it was multiplied by $150/316 = 0.4747$.

$$0.780 \times 0.4747 = 0.3703 \text{ g. arabinose} = 18.5 \% \text{ (approx.).}$$

As the total amount of pentose present was found to be 70 % the percentage of xylose may be roughly calculated as 51.5 %.

An estimation of the amount of mucic acid formed from the mixed sugars was now attempted in order to obtain some idea of the relative amount of galactose, and hence of mannose present. A modification of the Tollens method

described by Fernau [1909] was employed for this purpose. It was found that 4.89 g. of mixed sugar crystals gave 0.3640 g. of mucic acid, or

$$0.3640 \times 1.33 = 0.4841 \text{ g. galactose.}$$

If the sugar from which the mucic acid was obtained were pure galactose, this would represent 70 % of the galactose present, according to Fernau. The percentage of the sugar present would, therefore, be 6.9, approximately. In a mixture of sugars, however, the percentage of galactose obtained by the mucic acid estimation would probably be much lower, and besides it is not likely that mannose is present in any great amount, as it is found mostly in gymnosperms [Schorger, 1917]. Pinoff's test [1905] for fructose, gave a negative result.

It was not found possible to form either the hydrazone or the osazone of glucose from the mixed sugar crystals. On treating the crystals with diphenylhydrazine only the diphenylhydrazone of arabinose was formed. It is therefore assumed that glucose is not present.

SUMMARY.

Apparently the chief product of hydrolysis of the hemicellulose of American White Oak is *l*-xylose. According to Browne [1912, p. 553], xylan, with a little araban, makes up about 15–25 % of the dry matter of deciduous trees. Arabinose is apparently present in much smaller quantities than xylose in American White Oak. A rough quantitative estimation gave the amount of xylose yielded by the hemicellulose of this wood as 51.5 % and that of arabinose as 18.5 %.

Hexoses apparently form about 30 % of the products of hydrolysis. These have been identified as mannose and galactose. A mucic acid estimation of the galactose gave approximately 7 %, but as the estimation was made on the mixed sugars, the true figure is probably very much higher, as the amount of mannose present appears to be very small.

It is impossible to say in what combination these products exist in the wood. Probably in the form of an arabo-xylan and a manno-galaetan.

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LXII. PECTIN AND ITS HYPOTHETICAL PRECURSOR, "PROTOPECTIN."

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It has long been known that the expressed juice of unripe fruits contains very little pectin, and that when the crushed marc is subsequently extracted with cold water, only small, additional quantities of this substance are obtained. In contrast to this, when a soft, fully ripe fruit is pressed, the juice contains a considerable amount of pectin, and further and larger quantities may readily be obtained by extraction of the marc with cold water.

When, however, an unripe fruit is heated with dilute hydrochloric acid it becomes thoroughly disintegrated, and the liquid obtained by straining and pressing the resulting mixture is rich in pectin. This has led to the assumption that unripe fruits contain an insoluble compound which might be regarded as the parent substance of pectin, and which, by hydrolysis, during the process of ripening, or when heated with acid, becomes converted into ordinary, soluble pectin. To this insoluble compound the name "protopectin" was given by Fellenberg [1918], and it corresponds to the "pectose" of earlier investigators. It has, moreover, been assumed that the presence of this "protopectin" was a cause of the hardness of unripe fruits, and that its gradual change into soluble pectin was a feature of the process of ripening.

After having worked for some time with pectin, however, it occurred to the present author that the generally accepted explanation of the apparent deficiency of soluble pectin in unripe fruits might not be the correct one. To quote one observation: if it be attempted to filter a solution containing only a relatively small concentration (0.5 % or even less) of pectin through a reasonably efficient paper it will be found that, after a very few moments, filtration becomes extremely slow. The filtrate then collected contains only a very small proportion of pectin, the greater part of the latter substance being retained on the surface of the filter as a gel-like layer. Considerable quantities of distilled water may then, in course of time, be passed through this filter, when the pectin continues to be removed, but at an extremely slow and apparently constant rate. Now it seemed that conditions analogous to this might prevail in the case of hard, unripe fruit, where really efficient disintegration of the tissue is peculiarly difficult to attain, particularly when it is considered that

fruits contain products which, though soluble in alcohol, are insoluble in water, and would certainly hinder the access of the latter to the pectin occluded in the tissue.

In view of these considerations, and also taking into account the fact that no direct evidence of the existence of "protopectin" has ever been obtained, the following alternative explanation of the fact that fruits not fully ripe retain part of their pectin content in an apparently insoluble form, presented itself to the author: namely, that no such substance as "protopectin" exists, but, that the persistent retention of pectin in an apparently insoluble form by the tissue of incompletely ripe fruits is due partly to the presence of substances insoluble in water but soluble in alcohol, but more particularly to the great difficulty in attaining, by mechanical means, really efficient disintegration of the unripe tissue. In other words, it is suggested that the presence of "protopectin" is not a cause of the hardness of the tissue of unripe fruits, but that the behaviour of this tissue, described above, is the effect of its dense nature.

More than two years ago a few preliminary observations were made which tended to support this view. The material employed was a very finely ground sample of dried pomace (the press-cake from the cider press). Three quantities, each weighing 30 g., were exhaustively extracted in a Soxhlet apparatus; No. 1 with water only; No. 2, first with alcohol, and subsequently with water; whilst No. 3, after extraction with alcohol, was heated in an autoclave at 110° with dilute hydrochloric acid for one hour, and subsequently exhausted with boiling water. The amounts of pectin obtained from the three extracts were, respectively, 1.4 g., 1.6 g., 1.6 g. Had "protopectin" been present the last extract should have yielded the largest amount of pectin, whilst the inhibiting effect of the presence of alcohol-soluble substances is shown by the lesser amount of pectin obtained from the first extract.

It was therefore decided to conduct some experiments with unripe apple tissue, taking precautions to remove all the substances soluble in alcohol and also to ensure as complete a mechanical disintegration as was possible with the means available.

The variety of apple selected for this purpose was Bramley's Seedling. The fruit for the experiment described below was picked on August 29th, 1922, when it was far from being in a ripe condition. These apples, when pressed, yielded a juice which contained only a very small amount of pectin. A quantity of the fruit was peeled, deprived of all core tissue, thinly sliced, and then placed in alcohol, these operations being conducted as quickly as possible. The weight of fresh, sliced material taken was 286 g. The alcohol into which the sliced fruit had been placed was decanted, and replaced by fresh solvent, and this treatment was continued, at the ordinary temperature, so long as anything was dissolved from the apple tissue. The latter was then well crushed in a mortar, brought on to a filter, and well washed, first with absolute alcohol, and finally with ethyl acetate. After drying, first in the air, but subsequently in a vacuum over sulphuric acid, the material weighed only 8.9 g. This dry

material was then submitted to a very thorough grinding in a mortar and the whole of it passed through a sieve the meshes of which were approximately $\frac{1}{200}$ th of an inch square. The ground product then weighed 8.8 g. This finely ground tissue was then well stirred, at frequent intervals, for about 2 hours with 1300 cc. of cold distilled water¹, and subsequently allowed to settle for about 22 hours. The supernatant liquid was then decanted, and the process repeated. In all, 25 extractions were made. The pectin content of each of the last ten extracts was very small, and apparently constant. The residual, wet marc was then mixed with about a litre of alcohol, collected on a Buchner filter, well washed, first with alcohol and subsequently with ethyl acetate, pressed, and allowed to dry. The dry material was re-ground with fine, sifted sand² in a very thorough manner, so that the sand became crushed to microscopic, sharp-edged, flint-like fragments. After again passing the product through the above-mentioned sieve the extractions with water were repeated as before. In this case it was found that only ten extractions were necessary to reach the point where only a very small amount of pectin was removed. The marc was then washed with alcohol and ethyl acetate, and dried, as previously described, after which it was again very thoroughly ground with the finely comminuted sand which had passed through the sieve after the first re-grinding. On repeating the treatment with water, as before, only seven extractions were found necessary. At this stage the material was carefully examined under a microscope, samples being taken two minutes after it had been vigorously stirred with distilled water, some from near the surface of the mixture, and others from the lowest portion. The former showed only fragments of cell walls together with minute particles of silica, whilst the latter contained, in addition to silica, some small fragments of vascular tissue which has escaped complete disintegration, together with one or two groups of about three partially broken cells still adhering together. It was evident that disintegration had been very thorough, although not absolutely complete, and it was concluded that a small amount of soluble pectin would still be retained in the tissue. Nevertheless, it was not deemed necessary to continue the laborious process of grinding and extracting in order to obtain proof of the absence of "protopectin." The residual marc was therefore heated in an autoclave at 110° with *N*/20 HCl under the conditions described by Carré [1922] and subsequently extracted with distilled water four times, in the manner described. The fourth extract contained an amount of pectin that was scarcely detectable. The pectin present in all the four series of extracts, obtained as above described, was, in each case, directly estimated as such. After concentration under diminished pressure, each extract was treated with a large volume of alcohol, the pectin collected, dried in a vacuum and weighed. The method proposed by Carré and Haynes [1921] was not adopted, as their procedure represents

¹ In order to ensure that the dry powder would readily mix with the water it was found convenient in the first instance to moisten it with a little alcohol.

² Purified by extraction with acids followed by strong ignition.

as pectin any acid which might happen to be present and which yields a calcium salt insoluble in dilute acetic acid. The following is a summary of the results obtained by the present author:

Weight of fresh apple tissue 286 g.	Weight of ground and dried tissue after treatment with alcohol 8.86 g.	Volume occupied by dry ground material About 28 cc.
	Weight of pectin from 8.80 g. of dry tissue g.	Volume occupied by wet tissue on subsidence from water (approximate) cc.
1. After fine grinding	1.9347*	330
2. After re-grinding with sand	1.5104*	220
3. Again re-ground with sand	0.3238	110
4. Autoclaved with HCl	0.1005	115

* These two weights, particularly the first one, are very slightly greater than the actual weight of pectin obtained. This is because, during the decantation of the pectin solutions from the marc, a very small amount of the latter was unavoidably removed with the pectin solution, and it was not subsequently separated owing to the above-mentioned difficulty of filtering, through a good paper, solutions of pectin which are not very dilute. The liquids decanted after the third and fourth treatments, however, were each exactly filtered, so that the weights of pectin yielded by them are strictly comparable. Nevertheless, the total percentage of pectin calculated on the weight of fresh tissue taken appears to be high in comparison with results previously obtained. This is attributed, in part, to the efficiency of the grinding and extraction, but also, probably, to the unripe condition of the fruit, since fully ripe fruit from the same trees was found to contain at least 50 % more water.

The volumes occupied by the marc are given as supplying additional evidence of the change produced by the various treatments.

It is seen from the above results that, after the removal of the "soluble pectin" from the original, finely ground tissue, a further, and almost as great an amount may readily be dissolved by treatment with cold, distilled water when the material has merely been submitted to a process of more efficient mechanical disintegration. The amount of pectin subsequently obtained after treatment with acid is insignificant, and its retention by the marc is obviously due to the fact that quite complete disintegration of the tissue had not been attained, even after the drastic grindings that had been resorted to.

The only conclusion that can be arrived at is, therefore, that "protopectin" does not exist, but that all the pectin present in the apple occurs in the ordinary, soluble form. The problem of estimating pectin in fruits therefore appears to resolve itself, in the first instance, into one of attaining complete disintegration of the tissue. It seems likely that this would most readily be accomplished by resorting to the treatment with dilute HCl employed by Carré [1922] for the hydrolysis of the supposed protopectin.

After the commencement of this work a paper, referred to above, was published by Carré, dealing with the changes which occur in the "pectic" constituents of stored fruit. The conclusions arrived at during the present investigation offer a ready explanation of some of the results there described. In the first place, it is probable that the "considerable variations" in the pectin content of stored fruit observed by Carré were due, not so much to a large sampling error, as suggested by her, but to differences in the degree of mechanical disintegration of the various samples of tissue before extraction

of the "soluble pectin." The great deficiency of pectin found by her in the early stages of ripening would appear to be chiefly due to lack of efficient disintegration, which is especially difficult to attain when the fruit is in an unripe condition. No special precautions seem to have been taken by Carré to ensure thorough grinding, and, in fact, she appears to recognise that this had not been attained. Thus, on p. 708, when describing the estimation, by treatment in an autoclave at 110° with dilute HCl, of protopectin in tissue which was supposed to have been already deprived of soluble pectin, she states: "The material became thoroughly disintegrated during the process...." Then again, she states that "A series of estimations of pectin and protopectins carried out at regular intervals on the same apples, showed that a very definite relationship exists between them and that the changes in the two constituents tend to be equal and opposite in amount." This, also, is obviously another result of varying degrees of inefficient mechanical disintegration. Naturally, the more pectin that can be removed after the first process of partial disintegration, the less there is left to be removed when the product has been submitted to a process which ensures complete disintegration.

It was observed by Carré that after crushing, and thoroughly washing apple tissue with water, a small amount of pectin continued to be removed during several weeks, on further treatment with water. This behaviour is attributed by the present author to the slow rate of diffusion of pectin from insufficiently disintegrated tissue.

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LXIII. MECHANISM OF OXIDATION IN THE PLANT.

PART I. THE OXYGENASE OF BACH AND CHODAT: FUNCTION OF LECITHINS IN RESPIRATION.

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IN the ordinary guaiacum test for plant oxydases two types of reaction are met with according to the variety of plant examined. Several species, notably *Russula* and *Lactarius*, are capable of reacting directly with a fresh alcoholic solution of guaiacum resin with production of its characteristic blue oxidation product. On the other hand, the majority of plant species only give the guaiacum reaction in presence of hydrogen peroxide, or of such substances as peroxidised turpentine, etc. In an attempt to correlate these two types of reaction Bach and Chodat [1903, 1] suggested the existence of two enzymes in the plant oxidative system: (i) a substance which they termed "oxygenase," which has the property of fixing atmospheric oxygen in such a manner as to produce a peroxide, and (ii) a "peroxydase" which catalyses the decomposition of the peroxide, with liberation of active oxygen. The view of these authors has been rather generally, though not universally, accepted. It is admittedly questionable as to whether all the so-called direct oxydases are resolvable into the two types of constituents suggested. At the same time it will be realised that if plants which give only the indirect, or peroxydase, reaction are, in the ordinary course of metabolism, to effect oxidations at all comparable to that of guaiacum or of the other reagents usually employed, some substitute within the plant for the hydrogen peroxide which must be added in the ordinary laboratory test, will be admittedly essential.

Existence of Peroxides in Plant Juices.

The existence of peroxides in plant juices has given rise to a certain amount of controversy. De Clermont [1875] reported the existence of hydrogen peroxide in vegetable juices. His observation was supported by Griesmayer [1876], Béchamp [1882], and by Bert and Regnard [1882]. Bellucci [1878] could not confirm the observation of these authors by means of the ordinary chromic acid reaction. It is to be observed, however, that the chromic acid reaction, though sensitive for hydrogen peroxide, is rarely given by other peroxides. Generally speaking, it is a matter of no little difficulty to distinguish between hydrogen peroxide and peroxide derivatives when the substance in question is present in relatively small quantity. The reactions, therefore,

which the above authors attributed to hydrogen peroxide may possibly have been given, instead, by organic peroxides present in the cell-sap. Bach and Chodat [1902] showed that the juice of *Lathraea squamaria* contained a substance which turned potassium iodide-starch paper blue. It could be precipitated with 1 % baryta solution. On washing the precipitate and decomposing with weak acid the product was found to be non-reactive towards titanium sulphate, but continued to react with potassium iodide-starch paper. The solution gave no reaction for nitrous acid. They therefore concluded that the reactive substance present was a peroxide. Onslow, M. W. [1919, 1920] also brought forward evidence in favour of the formation of peroxides in various plant extracts.

Evidence obtained in the course of the present work indicates that a substance, or substances, of a peroxide nature is formed in plant extracts in contact with air. It is a matter of common experience that the outer portion of vegetable roots is found to give the direct oxydase reaction with guaiacum, while the inner portion of the root only gives the indirect or peroxydase reaction. If, for instance, a thin section be cut from the surface of a potato and the portion of the tuber so exposed be treated with fresh guaiacum tincture, an immediate intense blue coloration is obtained. If a tuber be cut in two through the centre, a solution of guaiacum poured over the fresh surface produces an immediate blue coloration only in the portion near the skin. Subsequent addition of hydrogen peroxide results in the production of a blue colour of nearly uniform intensity over the whole surface. It is frequently found that if a section of tuber, cut through the centre as in the last instance, be allowed to remain exposed to air for some time before treatment with guaiacum, a more or less uniform coloration over the whole surface is obtained. It is not, however, always a simple matter to demonstrate peroxide production in a plant tissue by this latter method. Moore and Whitley [1909] suggest that the occasional failure of this test is due to the destructive action of excess of oxygen on the peroxide first formed. Since, however, the test is nearly always positive in winter and frequently negative when tissues are examined in the summer, a more likely explanation would seem to be that at a time of relatively great vegetative activity the peroxide is used up as quickly as it is formed.

Various authors have suggested that the difference between the direct oxydase reaction and the indirect, or peroxydase, reaction is one of degree only, depending on the relative concentrations of a single oxidising enzyme. The fact that peroxydase enzyme preparations of very different concentrations are not known to show any difference in their manner of reacting with guaiacum would appear to be strong evidence against this view. When a vegetable extract has been made capable, by exposure to air, of directly causing the oxidation of guaiacum (without special addition of peroxide) the change accomplished is found to have taken place in a constituent other than the peroxydase. The following observations make this point clear.

Some freshly-peeled potatoes or mangold roots are finely minced and the pressed-out juice is treated with not less than five times its volume of rectified spirit. This ensures fairly complete precipitation of the peroxydase. After standing for a day the precipitate is filtered off. The peroxydase contained therein is purified somewhat by dissolving in water and reprecipitating. A solution of peroxydase so obtained gives no coloration when added to fresh guaiacum tincture. If a few cc. of the fresh alcoholic filtrate be diluted with an equal volume of distilled water and then added to the mixture no oxidation of the guaiacum will be found to take place. It will be found, however, on storing the two fractions obtained from the plant extract, in contact with air, that the alcohol-insoluble fraction shows no alteration in the manner of its reacting with guaiacum, while the alcohol-soluble fraction gradually acquires the power of reacting with guaiacum in presence of peroxydase. This alteration which takes place in alcoholic extracts of plants on storing in contact with air would seem to be the most reliable method of demonstrating the formation of peroxide from plant material. The percentage of alcohol (nearly 80 %) in the extracts is such as to eliminate bacterial action.

These results lend very considerable support to the Bach-Chodat view as to the dual nature of the oxidative system of the plants examined. The question arises as to what extent the classification by these authors of the production of peroxide as an enzyme reaction, as ordinarily understood, is justified. An investigation as to the nature of the substance concerned was therefore undertaken.

Nature of the Peroxide-forming Constituent of the Plant-cell.

Moore and Whitley [1909] in a study of oxidising enzymes demonstrated the presence of peroxides in plant juices and their formation by action of atmospheric oxygen. They disputed, however, the intervention of a special enzyme in the production of these peroxides, as had been suggested by Bach and Chodat. Onslow, M. W. [1919, 1920] asserted that the peroxide was produced by a derivative of catechol present in the plant, and that the fixation of oxygen to this body was catalysed by an "oxygenase" also said to be present. Prior to the publication of the Bach and Chodat theory Kastle and Loevenhart [1901] stated that oxydase is not a true ferment, but an organic peroxide. They explained its activity on Baeyer and Villiger's theory in that it acts as a carrier of oxygen in the same way as does benzaldehyde, but is not a true catalytic agent. The Baeyer and Villiger theory referred to is in reality that of Bach [1897]. In dealing with the well-known phenomenon of autoxidation Bach suggested that the substance undergoing autoxidation united with whole molecules of oxygen to form a peroxide. The peroxide thus formed, or the hydrogen peroxide in the so-called nascent state resulting from its decomposition, was held to be the active oxidising agent in such a case, for instance, as the oxidation of indigo simultaneously with the autoxidation of benzaldehyde or of oil of turpentine. A peroxide of benzaldehyde of the type suggested

by Bach was subsequently prepared by Baeyer and Villiger [1900]. It will be noted that Kastle and Loevenhart regarded oxydase as a single active substance. Their statement, that organic peroxides give an immediate blue coloration with guaiacum, could not be confirmed in the present work, other than in the case of benzoyl peroxide. Organic peroxides in general, like hydrogen peroxide, only cause a rapid oxidation of guaiacum in presence of a catalyst, such as various salts of manganese or iron, or in presence of natural peroxydase. The necessity for some such catalyst is at present fairly generally accepted¹.

In connection with the mechanism of the production of peroxide in the plant, it will be seen that the view of Kastle and Loevenhart, in which it is suggested that one of the plant constituents is of an autoxidisable nature is relatively more simple and in keeping with the facts than the assumption of a special ferment. The known autoxidisable organic compounds are invariably substances of an unsaturated nature. The production of a peroxide as an essential stage in autoxidation phenomena, though difficult to demonstrate in some cases, has, none the less, been confirmed in many instances. Thus Engler and Weissberg [1904] have succeeded in preparing, in a pure condition, peroxides of fulvene formed by the action of molecular oxygen. Harries and Muller [1902] describe similar preparations. In many other cases, though the actual peroxide has not been isolated, its presence can be shown by means of suitable reagents. Unsaturated substances present in oil of turpentine give rise to peroxide derivatives in presence of air. As is well known, aerated turpentine may be substituted for hydrogen peroxide in the ordinary guaiacum test for peroxydases. The same is true of aerated benzaldehyde or linseed oil, though in these cases but slight amounts of peroxide seem to be present.

M. W. Onslow [1919, 1920] claims to have shown that the blackening of plant tissues on injury is due to the oxidation of a substance which contains a catechol group, and it is stated that this same phenolic substance functions as the medium of peroxide production in plant tissues. The *ortho*-diphenol concerned has not been characterised further than by the green coloration given by certain plant extracts with ferric chloride. For the purpose of investigating the generalisations mentioned an examination was made in the present work of the root of the common mangold, *Beta vulgaris*. The mangold root, on mincing, first becomes brown and then intensely black on exposure to air. An extract of the root, prepared as described further on (Experimental Part, Section 3) was found to give a blue-black precipitate with a trace of ferric chloride. On adding excess of the salt a green coloration was obtained. On further examination, the extract was found to give many reactions charac-

¹ The guaiacum solution used in the present work was made by dissolving 0.5 g. of translucent resin in 5 cc. of boiling alcohol. An equal volume of water was then added. No coloration should be obtained on addition of a pure peroxydase; nor should any oxidation take place on addition of peroxide in absence of peroxydase. It will usually be found that only fresh solutions of the reagent fulfil both of these conditions.

teristic of the tannins. It is thus evident that the coloration obtained with ferric chloride is due to a substance of this nature. The substance present in the mangold appears to be a gallo-tannin, and the question arises as to whether the catechol derivative referred to above be not, in fact, a catechol tannin.

Peroxide-forming property of Tannin-fraction from the Mangold.

After allowing the tannin-containing extract to remain in the bottom of a stoppered Erlenmeyer flask for a couple of weeks it was found that on adding a quantity to a mixture of the peroxydase enzyme and fresh guaiacum tincture, a slight oxidation of the guaiacum took place. The solution was subsequently stored in a dark place for some months. A black amorphous substance was deposited. After two months the test with a mixture of peroxydase and guaiacum was found to give quite an intense blue coloration. At this stage, however, it was noted that the solution no longer gave any coloration whatsoever with ferric chloride. Quantitative study here will be necessary to determine definitely whether production of peroxide continues after the disappearance of the tannin reactions. It would appear from the qualitative study made that the production of peroxide tends noticeably to increase as the phenolic reaction disappears.

"Anti-oxygen" substances.

It will be seen from the foregoing that the view that catechol derivatives are the peroxide-forming substances of the plant is based on insufficient evidence. The inhibitory influence of phenols on the fixation of oxygen to autoxidisable substances, as recently studied by Moureu and Dufraisse [1922] renders this view of the function of phenols in the plant rather untenable. The results of these authors show that the addition of phenols or tannins to autoxidisable compounds prevents the formation of peroxide, and they ascribe to such substances the rôle of anti-oxydases or oxydation buffers (anti-oxygènes).

The Blackening of Vegetable Juices on Exposure to Air.

When the fresh tannin-containing fraction from the mangold root is mixed with a solution of the peroxydase fraction from the same root the blackening characteristic of the fresh juice is obtained on exposure to air. It was found, however, that the aqueous residue which remained after extraction of the tannin portion with ether-alcohol mixture likewise gave this blackening even to a much stronger degree. It was found on exhaustively extracting this aqueous portion with ether that the substrate of the blackening process still remained in the aqueous layer after the substance which gave the ferric chloride coloration was completely removed. This clearly indicates that the compound which gives rise to the black oxidation products must be other than that which gives the ferric chloride coloration. Closer examination of the blackening process led to the conclusion that the characteristic blackening of the juice of the mangold is, in fact, due to the action of tyrosinase on tyrosine. A study of the darkening of the juice of the potato led to a similar conclusion (see

Experimental Part, Section 2). Bertrand [1896] attributed the blackening of the sap of beetroot, potato and dahlia to the oxidation of tyrosine by tyrosinase.

The Peroxide-forming Constituent of the Potato.

The bulk of the evidence would seem to indicate that the oxygenase of Bach and Chodat is a substance of an autoxidisable nature. Experiments were consequently carried out with a view to the isolation of such substance or substances from the plant. The material chosen for special study was the potato tuber. In this particular isolation there existed the disadvantage that the substance sought for gave no direct characteristic reaction which would enable its location after a series of operations to be rapidly and easily ascertained. It was necessary to adopt the procedure of starting with material which gave no reaction for peroxide when treated with a mixture of fresh guaiacum tincture and peroxydase solution and subsequently carrying out operations as far as possible in absence of oxygen. It was thus possible to ascertain whether any particular fraction obtained during the course of the treatment contained a constituent of peroxide-forming properties by exposing a portion of it in solution to the action of air or oxygen and subsequently testing this with guaiacum-peroxydase mixture.

The experimental details of the method, as finally adopted, are given further on. Briefly, the method consists in finely mincing the tubers and in extracting for 2 hours with alcohol on the water-bath at 50–60°. The product is filtered and evaporated down nearly to dryness under reduced pressure. The aqueous residue in the flask is then extracted with ether. The ether extract is filtered and evaporated down to small bulk. It is then poured into a large excess of acetone. The precipitate having been allowed to settle, the acetone is filtered off. The precipitated material is purified by frequent repetition of the process of precipitation from ethereal solution by means of acetone. The substance thus obtained proved to be of phosphatide nature. The acetone washings from each precipitate were found, after standing some hours in presence of air, to develop the peroxide reaction towards guaiacum-peroxydase mixture. This phenomenon was observed even after repeated precipitation. After removal of all traces of ether and acetone in a vacuum, an alcoholic solution of the substance exposed to air also showed peroxide formation. The final product from the acetone precipitations was precipitated from alcoholic solution as a cadmium chloride double compound. On decomposing this the regenerated lipin continued to give evidence of peroxide formation in presence of air.

Peroxide-forming property of Potato juice associated with the Lipins.

From these results it is evident that the production of peroxide in the potato is intimately associated with the lecithin of the tuber. The substance from which the peroxide is derived may either be the lecithin itself or a compound intimately associated with it. While bearing in mind the latter possi-

bility, it may be pointed out that, generally speaking, lecithins are unsaturated substances owing to their containing unsaturated fatty acids in the molecule, and autoxidation is very generally associated with unsaturation. Erlandsen [1907] gives some extremely interesting details as regards the autoxidisable nature of a lecithin which he examined. The freshly prepared substance proved to have the formula $C_{71}H_{125}NP_2O_{21}$. After storage for some time in dry air it was found to have altered to $C_{71}H_{125}NP_2O_{30}$. Storage of the material for one month in an unevacuated desiccator resulted in an increase in weight of nearly 9 %. The iodine value of the fresh material he found to be 101; after combination with oxygen it was found to have fallen to 22. This change of iodine value appears to indicate alteration of the fatty acid radicles under the influence of atmospheric oxygen. Thunberg [1911] makes the observation that lecithin is oxidised in air by ferrous ammonium sulphate. It would thus appear to form a peroxide after the manner of benzaldehyde (Bayliss).

Function of Lecithins in Respiration.

Palladin and Stanewitsch [1910] pointed out an apparent relationship between the lecithins and plant respiration. They treated wheat embryos with organic solvents and then soaked them for a short time in water, estimating the amount of carbon dioxide evolved during definite intervals. They found that the respiration energy was lowest after treatment with those solvents which extracted most lipin. Vernon [1912, 1914] claims to have demonstrated the dependence of the action of oxydases in the animal organism on the cell lipins. He concluded that the effect of the oxydase is dependent on the lipin membrane which, he suggested, holds together the tissue oxygenase and peroxydase and makes possible their mutual enzymic activity. If, however, as the present research on the potato indicates, the lipin be itself an oxygenase the results of Vernon, as also those of Palladin and Stanewitsch, are readily explicable.

Lecithins are known to be of very general occurrence in animal and vegetable cells, and their function, or functions, has so far remained rather obscure. The property of lecithins of being able to combine readily with molecular oxygen to produce a peroxide would therefore seem to be of rather general importance from the point of view of biological oxidation. Dakin [1922] has pointed out the marked resemblance between oxidation within the animal body and oxidations effected in the laboratory by means of hydrogen peroxide. Kostytschew [1910] claims to have succeeded in oxidising certain degradation products of glucose by the simultaneous action on these substances of a vegetable peroxydase and hydrogen peroxide. Free hydrogen peroxide appears to be practically absent from both vegetable and animal tissues. It is known to be relatively toxic to both, and the ferment catalase, of very general occurrence; prevents its accumulation. According to Bach and Chodat [1903, 2] catalase is entirely without action on "oxygenase" or on ethyl hydroperoxide. It is thus evident that a substituted peroxide of the type of ethyl hydroperoxide

must be the active oxidising agent in the cell rather than free hydrogen peroxide. Such peroxides, as a rule, are found to be very weak oxidising agents and require the addition of a catalyst before oxidations such as that of guaiacum, benzidine, etc., can take place at all readily. In the plant the catalyst is usually a peroxydase. It yet remains to be definitely determined whether the lecithin of animal tissues is capable, by the production of peroxide, of causing the oxidation of other substances present¹. The well-known peroxydase reaction of haemoglobin with respect to guaiacum, benzidine, etc., is of interest in this respect, and suggests that haemoglobin or some closely related substance in animal tissues may be capable of fulfilling a catalytic rôle comparable to that of peroxydase in the plant.

Plant Constituents other than the Lipins which may function as "Oxygenases."

Unsaturated substances, as has already been pointed out, are very frequently found to be autoxidisable. It is thus reasonable to expect that unsaturated compounds, other than the lipins, present in a plant may, according to circumstances, function as "oxygenases." The formation of peroxide by oil of turpentine, itself a plant product, is well known. It appeared interesting, therefore, to submit to examination a number of other terpenes with a view to ascertaining whether they behave in a similar manner. Specimens supplied by British Drug Houses, Ltd., were examined as delivered. The following gave a strong blue coloration with guaiacum-peroxydase mixture: limonene, cumene, cedrene, phellandrene, terpineol, terebene, terpinol, linalol and carvone. Pinene and carvene gave no coloration on preliminary examination. On placing a small quantity of each in test-tubes and passing a current of oxygen through for about four hours they were found, however, to have acquired the property of causing the oxidation of guaiacum in presence of peroxydase. On the other hand, samples of caryophyllene, citral, citronellal, camphene and terpene hydrate, even after oxygen had been passed through them for eight to nine hours, gave scarcely any coloration with guaiacum-peroxydase mixture. Like pinene and carvene, a specimen of oleic acid gave no reaction for peroxide until oxygen had been bubbled through it for three to four hours. The production of peroxide by oleic acid is of especial interest in view of its frequent occurrence in the molecule of lecithin-like substances.

These observations on the occurrence of peroxide among the terpenes examined are provisional. It is possible that in individual cases where a positive reaction for peroxide was obtained, the reaction may be due to an autoxidisable impurity present; or on the other hand, in those instances where no peroxide appears to be formed, the negative result may be due to an inhibiting substance. The results as given are none the less interesting as indicating the ease with which the terpenes, as a class, give rise to peroxides. It appears quite likely, in consequence, that these substances are thus capable of taking part in the respiration of the plants in which they are found.

¹ A specimen of egg-lecithin in alcoholic solution was found, after several days' exposure to air, to acquire the power of oxidising guaiacum in presence of peroxydase.

EXPERIMENTAL PART.

1. *Preparation of Solutions of Peroxydase from the Mangold root and the Potato.*

The solutions of peroxydase employed in the course of the present work were invariably prepared from the root of the mangold. The most suitable variety of root for the preparation is that known as "yellow globe." The product obtained from the mangold is not a pure peroxydase, but contains a considerable proportion of tyrosinase. The same is true of that from the potato tuber. It would appear that most tyrosinase preparations so far studied give the peroxydase reaction with guaiacum, but whether this is due to admixture with ordinary peroxydase or a property of tyrosinase itself it is impossible to say.

For the purpose of preparing the enzyme solution the plant material is first finely minced. The juice is then pressed out from the minced material through a double layer of fine muslin, and is treated with about five times its volume of rectified spirit. The mixture is allowed to stand overnight. The precipitate which collects is then filtered off. It is purified somewhat by redissolving in water and reprecipitating the enzyme by the addition of more alcohol in approximately the same proportion as above. The precipitate obtained in this case is likewise allowed to settle. After again filtering off, it is dissolved in a quantity of distilled water approximately equivalent to one-fifth the volume of the original juice.

The solution of peroxydase thus obtained from the mangold root, or from the potato, is a clear liquid of slight brown colour. In presence of hydrogen peroxide it oxidises guaiacum, guaiacol, benzidine, *p*-phenylenediamine, α -naphthol, etc. In the absence of hydrogen peroxide it is without action on these substances. A mixture of peroxydase and one of these substances is consequently an excellent reagent for detecting hydrogen peroxide, either in the free condition, or combined as a peroxide derivative. In the course of the present work use has been made of a mixture of peroxydase solution and fresh guaiacum tincture for this purpose. For this test to be reliable it is essential that care be taken to use a guaiacum solution free from peroxide, otherwise the mixture of guaiacum solution and peroxydase alone will yield the characteristic blue colour. It is likewise essential to note that the substance under examination be incapable of oxidising guaiacum in the absence of peroxydase¹.

¹ Guaiacum is readily oxidised by ozone, nitrogen peroxide, free nitrous and nitric acids, permanganates and chromates. In each of these cases, however, the action takes place in the absence of peroxydase. In the case of solutions, therefore, where guaiacum is oxidised only when peroxydase is also added there is ample evidence for believing that the solution contains either hydrogen peroxide or other peroxides of similar constitution. The guaiacum reaction, as described, would thus appear to be a specific test for peroxides. This cannot be said of the potassium-iodide-starch test which has been commonly employed in dealing with these substances. Guaiacum-peroxydase mixture appears to be considerably more sensitive to organic peroxides than mixtures of peroxydase and other common oxydase reagents (such as benzidine etc.) which are sometimes employed.

2. *Blackening of the Juice of the Mangold and of the Potato due to the action of Tyrosinase on Tyrosine.*

The influence of tyrosinase on the blackening of the juice of the potato may be demonstrated as follows.

A quantity of potato tubers is finely minced and the pressed-out juice is quickly filtered or centrifuged. About 10 cc. of the clear juice is then transferred to a boiling-tube by means of a pipette, care being taken that the juice falls directly to the bottom and does not spill along the sides of the tube. The boiling-tube is then immersed in a water-bath at 75° and the juice is stirred with a thermometer. When the juice has attained the temperature of the water-bath the time is noted and heating is continued for 10 minutes at this temperature. The contents of the tube are then cooled.

It will now be found that although the contents of the tube still give a strong peroxydase reaction with guaiacum, no darkening in colour takes place on exposure to air. If a quantity of tyrosine or of *p*-cresol be added to a portion of the juice so heated, the characteristic colorations given by these substances in presence of tyrosinase are no longer obtained, although fresh potato juice gives this reaction. It is thus evident that the tyrosinase has been destroyed on heating. If now a solution of tyrosinase, prepared in the ordinary manner from any plant containing it, be added to another portion of potato juice which has been heated as described, the darkening in colour characteristic of fresh potato juice is again obtained. Addition of a pure peroxydase preparation, such as that from the horse-radish, produces no change. That the actual substrate in this blackening process in the case of potato juice is free tyrosine is rendered very probable by the isolation from the potato of considerable quantities of this amino acid in the free condition. The substance was isolated incidentally in another portion of this work (see Section 6, Experimental Part) and considering that it was obtained in an alcohol-ether extract of a concentrate of the juice, the yield of 0.3 g. from 2½ kilos. of fresh tuber is very probably far from quantitative.

A similar destruction of the tyrosinase in the mangold root results in the prevention of the blackening of the juice. The tyrosinase of the mangold is usually found to be somewhat more resistant to heat than that of the potato, and a rather longer period of heating than 10 minutes at 75° is found necessary to complete its destruction in some samples. The peroxydase is also relatively more resistant to heat in this case than in that of the potato.

3. *Study of the Tannin of the Mangold root.*

The alcoholic filtrates obtained in the preparation of the peroxydase of the mangold were now studied in connection with the suggestion made by Onslow, M. W. [1919, 1920] that the peroxide-forming constituent in the plant as well as the substrate in the blackening process of the juice was a derivative of catechol. The alcoholic solution (six litres) obtained after removing

the peroxylase-tyrosinase fraction of the juice was concentrated *in vacuo* at 40–50°. When all of the alcohol had been removed and the aqueous residue suitably reduced in bulk the distillation was stopped. The residue was then extracted with ether containing about 20 % of alcohol. The ether was distilled off from the extract. A residue consisting of a solution in a mixture of water and alcohol was thus obtained. With ferric chloride it gave a blue-black coloration or precipitate, which disappeared on adding excess of the salt, yielding a green solution. On submitting the extract to further examination the following reactions were observed:

- (i) Addition of alkali causes the solution to darken in colour.
- (ii) With a solution of ammonium picrate it gives a red colour, which changes to green.
- (iii) On adding a little sodium sulphate, and then treating with a dilute solution of iodine a purple-red colour results.
- (iv) With a solution of potassium cyanide scarcely any change takes place.
- (v) A purple colour is obtained on adding, first, ammonia, and then nitric acid, to the solution.
- (vi) On treating 2 cc. of the solution with three drops of 20 % thymol solution and then adding 3 cc. of strong sulphuric acid a deep red coloration is obtained.

(vii) Lime-water gives a greyish precipitate which rapidly turns blue.

These reactions are characteristic of the tannins, and it is thus evident that the coloration given by ferric chloride is due to a substance or substances of this type. It is interesting to note, moreover, that tannins, as a rule, are said to have an inhibiting influence on oxydase action.

4. Peroxide-forming character of Alcoholic Extract of Plants, and Action of Phenols thereon.

The production of peroxide by a substance in plant tissue may best be demonstrated by exposing an alcoholic extract of the tissues to air. An alcoholic extract for the purpose may conveniently be prepared as follows.

Fresh mangold roots, or a quantity of potato tubers, are peeled and then finely minced. The minced material is quickly strained through several layers of muslin, 500 cc. of the juice thus obtained are then added to 2 litres of rectified spirit. The mixture is well shaken and after being allowed to stand overnight, is filtered. The alcoholic extract thus obtained produces no change when added to a mixture of peroxylase and fresh guaiacum tincture. In making the test 5 cc. of the alcoholic plant extract is diluted with an equal volume of distilled water before adding it to a mixture of peroxylase, prepared as in Section 1, and a few drops of a 5 % solution of guaiacum in 50 % alcohol. The dilution of the extract with water is necessary to prevent precipitation of peroxylase. On storing the alcoholic extract in presence of air, no change in its behaviour relative to a guaiacum-peroxylase mixture is observed till after a certain length of time. In the case of an extract of potato, 50 cc. of solution in an uncorked

250 cc. flask, placed so as to be exposed to daylight, only began to oxidise guaiacum-peroxydase mixture after two days' exposure. An extract of the mangold exposed in a similar manner required over a week's exposure before the presence of peroxide could be detected. There is thus evidence of a certain latent period in the production of peroxide, and this is probably due, in part at any rate, to the presence of phenolic substances such as tannins. The inhibiting influence of such substances on the course of autoxidation has been demonstrated by Moureu and Dufraisse [1922]. In the course of normal plant metabolism the proportion of these inhibiting substances present is probably controlled by the peroxydase, the known oxidising power of the latter enzyme relative to phenolic substances being responsible for its being commonly classed as phenolase.

The rate at which peroxide is produced in the plant alcoholic extracts appears to be very much accelerated by the action of light. Light, however, is not essential to the production of peroxide, since extracts stored in the dark were also found to have acquired it. As might be expected, the free admission of oxygen has also a marked accelerating influence.

5. *Action of the "Anti-oxygènes" of Moureu and Dufraisse on the production of Peroxide in Plant extracts.*

The following table shows the influence of traces of some common phenols on the rate of peroxide formation in alcoholic extracts of the mangold, prepared as described in Section 4.

100 cc. alcoholic extract treated with phenol in proportion of 1 : 100,000	Test with guaiacum-peroxydase mixture after one week's exposure to air	Test with guaiacum-peroxydase mixture after two weeks' exposure to air
1. Quinol	No coloration	Faint blue coloration
2. Gallotannic acid	Blue " coloration	" " " " coloration
3. Pyrocatechol	Blue coloration	Very " " " " coloration which quickly faded
4. Control	"	Strong blue coloration

The inhibiting influence of traces of quinol and of gallotannic acid on peroxide formation is seen to be distinctive. That the action of these substances is to prevent the production of peroxide is shown by the fact that subsequent addition of peroxide to the solutions containing them, after treatment with guaiacum and peroxydase, gives rise to the usual intense blue colour. Pyrocatechol appears to be without inhibiting action in the dilution employed. In the case of potato extract, *p*-cresol was likewise found to be without inhibiting action when added in traces similar to the above. On the other hand, a trace of pyrogallol was found to be quite effective in preventing peroxide formation.

6. *Preparation of the Peroxide-forming Constituent of the Potato.*

The method by which this substance was first prepared consisted in finely mincing 2½ kilos. of freshly-peeled potato tubers. The minced material was then added to 7½ litres of rectified spirit and the mixture was allowed to digest in the cold for 24 hours. The solid matter was then filtered off on the Buchner

funnel. The filtrate was evaporated down *in vacuo* at 40–50° until 400–500 cc. of aqueous residue remained. A quantity of waxy material separated. This was removed by thorough extraction with ether. This ether extract was subsequently found to contain the bulk of the peroxide-forming constituent of the tuber. The aqueous portion remaining after the ether extraction was then shaken with a mixture of equal parts of alcohol and ether. On concentrating this extract somewhat, a yield of 0.3 g. of free tyrosine was found to separate. That this amount of tyrosine is by no means indicative of the total amount of this amino acid present in the original tuber is evident from a consideration of the solubility of tyrosine in the solvents employed, as also from the fact that tyrosine continued to separate slowly, in a rather impure condition, from the aqueous solution remaining after the extraction. The ether extract obtained above from the original concentrate was then worked up. On distilling off the ether a wax-like material was obtained. This was redissolved in 15–20 cc. of ether. On addition of about 250 cc. of alcohol a light yellow solid was precipitated. The precipitate was redissolved in a small quantity of ether and then poured into about a litre of acetone, with stirring. A precipitate, more nearly colourless than that given by alcohol was thus obtained. On allowing the acetone washings from this material to remain exposed to air for some hours and then testing them with peroxydase-guaiaecum mixture a blue colour resulted, indicative of the formation of peroxide in the liquid. The precipitate obtained with acetone was again redissolved in a small quantity of ether and again poured into a large excess of acetone (500–600 cc.). This method of purification was repeated six times. In each case the acetone washings continued to give a peroxide reaction after exposure to air for some time. The final product, on drying off the acetone, became quite brown in colour, and contracted to a wax-like mass. On drying it in a vacuum desiccator it became quite brittle and could be easily reduced to powder. It proved to be relatively slightly soluble in alcohol. In alcoholic solution production of peroxide was also noted. About 0.2 g. of the substance was dissolved in alcohol by warming to 50–60°. 5 cc. of a saturated alcoholic solution of cadmium chloride were added. After standing for some time the precipitate which separated was filtered off. It was placed in a beaker and about 20 cc. of ether added. After acidifying with a drop of 10 % hydrochloric acid, the cadmium was precipitated by hydrogen sulphide and the sulphide was filtered off. Excess of hydrogen sulphide was then removed with a current of air and the ethereal solution remaining was washed by shaking in a separating funnel, first with a saturated aqueous solution of sodium bicarbonate and finally with distilled water. The remaining ethereal solution was then poured into excess of acetone. The product thus obtained continued to show peroxide-forming properties. After prolonged action of air it was found capable of liberating iodine from potassium iodide, even in the absence of acid.

It is evident from these results that the formation of peroxide is due to the autoxidation of a lecithin-like substance. It has been pointed out by

various workers on plant lecithins that these substances are not very readily extracted from the tissues by cold solvents. Schulze and his co-workers, who investigated many lecithins of this type, carried out their extractions with alcohol at 50–60°. The extraction of a further quantity of lecithin from the potato tuber was accordingly carried out as follows.

Three kilos. of fresh, finely minced tubers were treated with five litres of 90 % alcohol and the mixture was digested on the water-bath for two hours. To prevent autoxidation at this temperature a current of nitrogen was passed through the liquid during the digestion. The extract was then pressed out from the solid material through a couple of layers of fine muslin. The liquid thus obtained was cloudy, owing to solid matter in suspension. On allowing it to stand for about a quarter of an hour the solid material readily settles down. The liquid may then be decanted and the remainder filtered on a Buchner funnel. The liquid was evaporated down as in the previous preparations and the residue was then thoroughly extracted with ether. After dehydrating the extract with anhydrous sodium sulphate the liquid was filtered and the greater part of the ether was distilled off. The ethereal solution remaining (about 50 cc.) was then added to a litre of acetone. The liquid was allowed to stand in the dark for about an hour and the lecithin which separated out was then filtered off and dried in a vacuum desiccator. The yield of lecithin thus obtained from three kilos. of fresh tuber was 1.6 g. The substance thus obtained may be further purified as in the previous preparation.

A specimen of the substance obtained in the present work was submitted to hydrolysis for the purpose of characterisation. 0.5 g. of the substance was boiled for five minutes with 12.5 cc. of 6 % sodium hydroxide solution. 2 cc. of glacial acetic acid were then added, and boiling was continued for a further minute. The acid liquid was allowed to cool. It was filtered through a moistened filter paper. The presence of phosphate in the filtrate was proved by precipitates given by ammonium molybdate and magnesium sulphate. By treatment with a solution of iodine in potassium iodide, no evidence of the production of "Florence crystals" said to be characteristic of choline [Struve, 1900] could be obtained, but microscopic crystals unlike those obtained from choline were precipitated. These may possibly be due to betaine, which substance is said to replace choline in some plant phosphatides. It is hoped in further work to continue the study of this phosphatide.

SUMMARY.

The present work deals with the nature of the "oxygenase" in the Bach and Chodat theory.

It is shown that plant juices, on exposure to air, form peroxides. Alcoholic extracts of plant tissues, containing a sufficiently high content of alcohol to prevent bacterial action, are found to serve best for demonstrating this production of peroxide.

The formation of peroxide by the action of atmospheric oxygen on plant

extracts appears to be markedly influenced by the addition of certain phenolic substances such as quinol or gallotannic acid. Minute traces of such substances appear usually, though not always, to prevent the fixation of oxygen.

The suggested relation of catechol to the plant oxydase system is discussed. It is shown that the blackening of aqueous extracts of the potato and of the mangold is due, not to the oxidation of a catechol derivative under the influence of peroxydase, but to the action of tyrosinase on tyrosine. A detailed examination of the extract of the mangold root which gave a ferric chloride coloration revealed the fact that the reactive substance present was a tannin. The tannins, in general, are said to have an inhibiting influence on oxydase action.

There appears to be no definite evidence that the production of peroxide in the plant is due to enzyme action; it seems more likely that the formation of peroxide is due to the presence of an autoxidisable substance in the tissues. A substance of this nature isolated from fresh potato tubers was found to bear a close relation to the lipins. In contact with air or oxygen a solution of this substance acquires the property of causing the immediate oxidation of guaiacum in presence of peroxydase. It would thus appear that the so-called oxygenase of the potato is in reality an autoxidisable lecithin-like substance.

A study of the terpenes as possible "oxygenases" in the plants in which they occur would be of interest. It is shown that these compounds as a class are capable of combining with oxygen in such a manner as to cause the oxidation of guaiacum in presence of peroxydase.

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LXIV. THE PENETRATION OF ELECTROLYTES INTO GELS.

V. THE DIFFUSION OF MIXTURES OF CHLORIDES IN GELS.

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INTRODUCTION.

CONSIDERABLE emphasis has been laid during recent years on the analogies between colloidal systems and living cells in regard to their behaviour towards water and dissolved substances and it has become evident that the study of colloidal systems must throw light on the constitution and behaviour of living tissues. It is therefore of interest to enquire whether anything analogous to the phenomenon of antagonism between ions in their entrance into living tissues is exhibited in the penetration of electrolytes into gels.

Among the many antagonistic actions recorded for various plant tissues, those between different chlorides were among the earliest, and are perhaps now the best, known. Thus a very definite antagonism between sodium and calcium chlorides, between potassium and calcium chlorides, and between potassium and magnesium chlorides, and even between chlorides of the univalent metals, has been recorded by different writers [see Stiles, 1923, 1]. Experiments were therefore made on the diffusion of mixtures of chlorides through gels of agar-agar and gelatin with a view to determining whether any mutual hindrance to the penetration of salts through the gel resulted from the presence of one salt along with the other, such as might be compared with the antagonistic effects observed in living systems.

METHOD.

The measurements were made by means of the indicator method already described [Stiles, 1920; Adair, 1920]. In the earlier experiments, carried out four years ago, the original crude "half test-tube" method was employed. Subsequently, the refined technique recently described in detail [Stiles, 1923, 2] was employed, and coefficients of diffusion calculated from the measurements made.

Two gels were used: a 1 % agar-agar gel made from shred agar-agar, and a 10 % gelatin gel. In both cases the material was washed with distilled water before use until the washing water was free from chloride. The gels contained silver nitrate as indicator, which was present in a concentration of 0.005N.

The earlier experiments were conducted in a dark cupboard in the laboratory without temperature control, but the results obtained in any one series are strictly comparable as all the experiments in a series were carried out together. The latter was also the case with the later more exact experiments, but these were carried out in water-baths maintained at constant temperature by means of gas-mercury-toluene thermo-regulators.

Experiments were conducted with a number of pairs of chlorides, the two chlorides of the pair being present in a variety of proportions, but in all cases the solutions were normal as regards chloride. In the calculation of the coefficient of diffusion the solutions are assumed to be normal solutions, as they are such in regard to chloride, and Adair's second and more exact formula is used. For the purposes of the calculation the coefficient of diffusion of silver nitrate in the gel is taken as 10^{-5} sq. cm. per second [cf. Adair, 1920]. An error of 50 % in this does not alter appreciably the final values obtained for the coefficients of diffusion of the chlorides.

EXPERIMENTAL RESULTS.

1. *Agar-agar.*

The diffusion of the following mixtures was examined by the half test-tube method: sodium chloride and calcium chloride, potassium chloride and calcium chloride, potassium chloride and magnesium chloride, and potassium chloride and sodium chloride. The experiments were carried out in duplicate. Each tube contained a length of 7 cm. of gel surmounted by the same length of solution of diffusing salt. Solutions of the pure salts and solutions containing varying relative proportions of the two chlorides were used, but in no case could any indication of a hindrance to diffusion of the salt in a mixed solution be discovered. The penetration was measured after a number of different times; the results obtained for measurements made after diffusion had proceeded for about 27 hours are recorded in Table I. Similar results were obtained after both shorter and longer periods.

Table I. *Penetration of chlorides into gels of 1 % agar-agar from pure and mixed solutions.*

Relative proportions of chlorides First named : second named	Penetration in centimetres			
	NaCl + CaCl ₂ 27.3 hrs.	KCl + CaCl ₂ 27.8 hrs.	KCl + MgCl ₂ 27.25 hrs.	KCl + NaCl 27.63 hrs.
10 : 0	4.19	4.60	4.59	4.59
9 : 1	4.15	4.525	4.50	4.56
8 : 2	4.15	4.525	4.49	4.52
7 : 3	4.165	4.505	4.43	4.51
6 : 4	4.09	4.43	4.40	4.49
5 : 5	4.08	4.41	4.32	4.42
4 : 6	3.965	4.32	4.29	4.42
3 : 7	3.95	4.23	4.15	—
2 : 8	3.94	4.13	4.09	4.345
1 : 9	3.81	4.01	3.915	—
0 : 10	3.76	3.79	3.655	4.21

Although the method does not permit of the calculation of the coefficients of diffusion, and although the measurements on which each value for the penetration is based are few in number, it is clear from these results that there is no antagonism between the chlorides employed in regard to their diffusion through agar-agar. Nevertheless one series of experiments, that in which mixtures of calcium and sodium chlorides were employed, was repeated by the more exact method and the coefficients of diffusion calculated in each case, assuming that each solution was a normal chloride solution. The values for the coefficient of diffusion are recorded in Table II. They confirm the conclusion drawn from the experiments with the cruder "half test-tube" method as to the absence of any hindrance offered by the presence of one chloride to the diffusion of another, for as the proportion of calcium chloride in the mixture is increased there is a progressive lessening in the value of the coefficient of diffusion.

In order to indicate more clearly the relation between the composition of the solution and the diffusivity the results obtained are shown graphically in

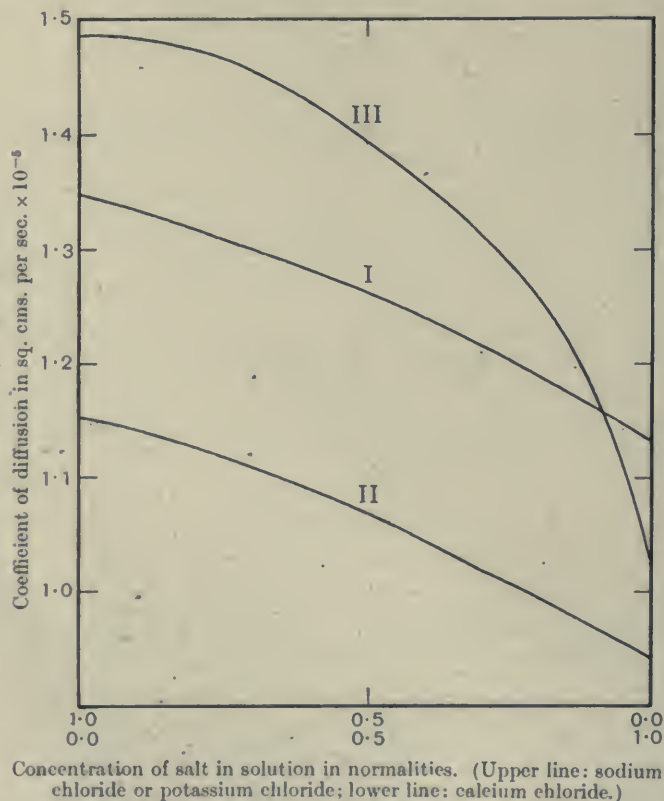


Fig. 1. Curves to illustrate the diffusion through gels of chlorides in pure and mixed solutions
 I. Sodium and calcium chlorides in 1 % agar-agar.
 II. Sodium and calcium chlorides in 10 % gelatin.
 III. Potassium and calcium chlorides in 10 % gelatin.

Fig. 1. These results thus expressed show that so far from there being anything in the nature of a hindrance to diffusion in a mixed solution, the diffusivity in a mixed solution is actually slightly greater than would be expected from the values of the diffusivity of the pure chlorides, as the curve connecting the value of the coefficient of diffusion with the composition of the diffusing solution is concave to the axis of the composition of the diffusing salt and not a straight line.

2. Gelatin.

Determinations of the coefficient of diffusion of sodium and calcium chlorides and of mixtures of the two in 10 % gelatin were made by the more exact method, the temperature of experiment being $21.5^\circ \pm 0.5^\circ$. A similar series of experiments was carried out with potassium chloride and calcium chloride as diffusing salts at a temperature of 21.5° . As before, all the solutions employed were normal in regard to chloride. The results are shown in Table II, and also graphically in Fig. 1. The same phenomenon is obvious here as in the diffusion of mixtures of chlorides through agar-agar, namely, that the rate of diffusion of the mixed chlorides is always a little higher than would be expected from the values of the coefficients of diffusion of the respective salts in pure solutions.

Table II. *Penetration of chlorides into gels of agar-agar and gelatin from pure and mixed solutions. (Concentrations of chloride always normal.)*

Relative proportions of chlorides First named : second named	Coefficient of diffusion in sq. cm. per sec. $\times 10^{-5}$		
	NaCl + CaCl ₂ in agar-agar 20.5°	NaCl + CaCl ₂ in gelatin $21.5^\circ \pm 0.5^\circ$	KCl + CaCl ₂ in gelatin 21.5°
10 : 0	1.347	1.151	1.488
10 : 1	—	—	1.476
9 : 1	1.334	1.141	—
10 : 2	—	—	1.481
8 : 2	1.317	1.131	—
10 : 4	—	—	1.457
7 : 3	1.297	1.106	—
5 : 5	1.269	1.073	1.393
3 : 7	1.210	1.012	—
4 : 10	—	—	1.305
2 : 10	—	—	1.239
1 : 9	1.166	0.969	—
0 : 10	1.130	0.940	1.024

It does not appear easy to find a simple explanation of these results. Although the coefficient of diffusion no doubt increases with increase in the relative number of electrolytically dissociated molecules and hence with dilution, as has been shown to be the case for gels [Stiles, 1923, 2] as well as for free diffusion in water, and although both of the two constituent salts in any one of the mixtures employed are in less than normal concentration, yet the presence of the other salt will depress the degree of dissociation so that an appreciable rise in the value of the coefficient of diffusion is scarcely to be expected on this ground.

A decrease in viscosity of the gel would result in an increase in the rate of diffusion, but the results obtained by Fenn [1916], if rightly interpreted, suggest that in mixtures of salts such as those employed in the present investigation, the viscosity is greater than in pure salt solutions of the same concentration.

In the present state of our knowledge it would not be profitable to discuss this matter further. It is, however, clear from the investigation here recorded that the antagonistic effects observed with living tissues cannot be attributed to mutual interference between the antagonising salts in their diffusion through any gel layer in the cells of the tissues.

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LXV. BROMOXYLENOL BLUE.

A TRUE-NEUTRALITY POINT INDICATOR.

By ABRAHAM COHEN (*London*).

(Received June 17th, 1923.)

In a previous article on xylenol blue, synthesised, and described in this Journal, [1922, 1] by the writer it was shown that the double working ranges of utility coincide with those of thymol blue. The pair of indicators thus share with certain other pairs of homologous indicators the property of anomalous unshifted p_H ranges. Advantage was taken of this property in the case of xylenol blue to obtain a solution of twice the intensity of thymol blue.

When the p_H range of the bromo-derivative of xylenol blue was investigated in the same manner as that of the unbrominated indicator, and compared with bromothymol blue in identical concentrations and strengths of indicator in buffer solutions of p_H 6.0–7.6, the series in each case was similar. The analogy of xylenol blue with thymol blue in respect of range of p_H is thus continued on bromination.

It is easier, however, to brominate xylenol blue than thymol blue, and this was accomplished by adding in the cold, with constant shaking of the reaction flask, 3.1 parts bromine to 1 part of xylenol blue suspended in 10 parts glacial acetic acid. After standing overnight, the pale pink crystals were thoroughly drained by suction and recrystallised from dry boiling toluene, in which the product is much more soluble than the similarly prepared bromocresol purple. It separated white and crystalline on cooling. Submitted to analysis, the product was found to be a *dibromoxylenol sulphonephthalein*.

0.3400 g., fused with caustic potash and sodium peroxide, gave 0.2250 g. silver bromide and 0.1432 g. barium sulphate.

S	5.76 %	calculated for	$C_{23}H_{20}O_5Br_2S$	5.63 %
Br	28.2 %	„	„	28.15 %

Bromoxylenol blue, like bromothymol blue, can have its working range halved by the addition of bromocresol purple, as shown in an earlier paper by the writer [1922, 2]. The resulting mixed indicator can then be used where a blue end-point of Sørensen value about 6.8 is desired in a titration.

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LXVI. BLOOD ENZYMES. III. ON THE GLYCOGENOLYTIC ACTIVITY OF MAMMALIAN SERA— WITH REMARKS ON SERUM TOXICITY.

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(Received June 20th, 1923.)

THE present communication treats of the enzymes, or enzyme complex, of the blood responsible for the breaking down of glycogen by hydrolysis. The end-products of glycogen hydrolysis being either maltose or glucose, according to the hydrolytic agents employed, it is assumed that at least two distinct enzyme systems are involved in the complete breaking down of glycogen to glucose: one, *glycogenase*, whose function would be to transform the glycogen first into maltose; and the other, *maltase*, concerned with the transformation of the maltose into glucose.

Now, it has been shown by us [Compton, 1921] that mammals differ considerably, from the species point of view, in the content of their blood in the last-mentioned ferment. For instance, when tested *in vitro*, the sera of man, the cat, the rabbit and the guinea-pig are entirely devoid of maltose splitting power; whilst sera from the dog, the horse, the ox, the goat and the pig—particularly the last-mentioned mammal, the blood of which is very rich in the ferment—normally contain *maltase*. Thus it comes about that, in terms of the enzyme content of their blood, there are two groups of mammals: a *positive blood maltase* group, and a *negative blood maltase* group.

On the other hand, the sera of all mammals which we have thus far investigated are capable of breaking down glycogen, with the production of either maltose or glucose. Whether the end-product of the hydrolysis of glycogen by mammalian blood serum is glucose, or maltose, must evidently depend upon the blood maltase group to which the animal belongs.

The results which follow concern the hydrolysis of glycogen by the sera of various mammals in a 16 hours' action at approximately 46–47°, the mammalian species studied comprising representatives of the two groups previously defined by us: (1) the *maltase* group: the dog, the horse, the ox, the pig, and the sheep; and (2) the *non-maltase* group: the cat, the rabbit, the monkey, and man.

It will be seen from the results set forth that here again, as in the case of *maltase*, mammals differ among themselves in the content of their sera in *glycogenolytic power*—as measured by the amount of glycogen hydrolysed in a given time at a given temperature by a given quantity of serum—the activity, moreover, being fairly characteristic of the species.

EXPERIMENTAL.

Preparation of Glycogen. Twenty-two rabbits were fed during a week largely on carrots, and the animals were then killed. The livers were removed immediately, minced, and plunged into an equal weight of 60 % potassium hydroxide, and the mixture heated up to boiling for 5 hours. An equal volume of water was added, and the entire mixture allowed to cool. Contrary to the methods usually described, it was now precipitated directly without previous filtration, by the addition of one and a half volumes of alcohol. After standing overnight, the precipitate was collected by centrifugation, and washed well with 70 % alcohol. The resulting, fairly white, precipitate was now heated on the water-bath for $1\frac{1}{2}$ hours with about six times its own weight (moist) of water to dissolve it, and was then filtered by suction while still hot through two layers of starch-free filter paper on a Buchner funnel. The solution thus obtained was rapidly cooled under the water-tap, and neutralised with concentrated hydrochloric acid, and about 3–5 cc. excess of acid added to precipitate any extraneous protein matter. The solution was next filtered anew through a Buchner funnel, an olive-green residue and a whitish opalescent solution resulting. The filtrate, and washings, some $2\frac{1}{4}$ litres in all, were poured into 4 litres of 95 % alcohol and the mixture allowed to stand overnight, the resulting white flocculent precipitate being collected and washed with 60 % alcohol. The precipitate (moist) was redissolved in ten times its weight of distilled water, and precipitated a third time with $1\frac{1}{2}$ volumes of alcohol, the opalescent solution being poured into the alcohol. Next morning, the precipitate was collected by centrifugation, washed twice with 70 % alcohol, and then with absolute. It was now dried in a vacuum over sulphuric acid. 120 g. of the dried pure product were thus obtained.

Complete Hydrolysis of Glycogen—its Glucose Content. According to Pflüger [1901], hydrolysis by 2·2 % hydrochloric acid on the water-bath for 3 hours completely transforms glycogen into glucose, and he utilised this as a method for estimating glycogen in tissues. In Pflüger's method the weight of glucose found after hydrolysis is multiplied by 0·927 to find the corresponding quantity of glycogen.

30 mg. of the foregoing specimen, when completely hydrolysed by prolonged heating with 2–3 % hydrochloric acid on the water-bath gave as the result of four experiments the following glucose numbers in mg., the latter being estimated by Bertrand's method: 29·8, 28·6, 29·2, 28·4—mean 29·0. This signifies that 100 mg. of the specimen when completely hydrolysed would yield 96·7 mg. approx. of glucose. From this it follows that 96·7 mg. of glucose found, as the result of the enzymic hydrolysis of 100 mg. of the specimen, corresponds to 100 % hydrolysis when the end-product is glucose. In other words, under such conditions 1 mg. of glucose found represents $\frac{100}{96.7} = 1.034$ % hydrolysis.

Again, since 1 mg. of glucose is the equivalent of $\frac{342}{360} = 0.95$ mg. of maltose, it will be evident, when the end-product is maltose that 1 mg. of maltose found as the result of the enzymic hydrolysis of 100 mg. of the specimen will represent a percentage hydrolysis of $\frac{100}{97.6 \times 0.95} = 1.09$ approx.

Method of Procedure. The method of determining the glycogenolytic activity of the different sera investigated was as follows: 100 mg. of glycogen were carefully weighed out into each of four, or more, clean dry test-tubes, and after adding 2 cc. of redistilled water to each tube, the latter were heated for about 2 minutes in boiling water to dissolve the glycogen. The tubes were rapidly cooled and the contents of each completed with water and varying doses of serum to 5 cc. Three drops of toluene were added, the tubes plunged into a water-thermostat regulated about 46–47°, and the tubes having acquired the temperature of the thermostat were closed with clean sterile corks. After incubation for 16 hours, the corks were removed and the enzyme action stopped by heating for 7 minutes in boiling water. When cold, the contents of each tube were diluted to 50 cc., and 20 cc. were withdrawn for a sugar determination by Bertrand's method. The percentage of glycogen hydrolysed was then calculated in terms of glucose, or of maltose, by the use of one or other of the preceding factors, according as the serum under investigation appertained to a positive, or a negative blood maltase group of mammals.

BLOOD MALTASE POSITIVE GROUP.

Under this category, sera from the dog, the horse, the pig and the sheep were investigated.

Dog. Three dogs were investigated (2-P, 3-P and 4-P). The animals were, as a rule, in the fasting condition for 24 hours previous to withdrawal of the blood for testing. The animal being immobilised, the blood was collected from the jugular vein into a sterile syringe, and centrifuged immediately to obtain serum for the tests.

The animal 2-P was examined, as to the richness of its blood in glycogenolytic activity, on three separate occasions; 3-P twice; and 4-P twice. The results obtained are set forth in Table I:

Table I.
Glycogen hydrolysed %, by the blood serum of the following dogs:

Dose of serum employed	2-P			3-P		4-P	
	1	2	3	1	2	1	2
0.1	29.2	23.7	—	23.7	—	33.0	—
0.15	—	—	34.4	—	35.6	—	47.0
0.2	40.8	37.7	—	35.2	—	53.0	—
0.3	—	—	50.9	—	45.4	—	65.1
0.4	52.2	52.3	—	42.9	—	69.3	—
0.6	—	—	66.6	—	60.5	—	69.8
0.8	65.9	66.7	—	57.6	—	71.8	—
Age of serum	2 days	Fresh	Fresh	2 days	Fresh	1 day	Fresh
Temp. at beginning and end of exp. °C.	47.4–46.8	47.5–46.9	47.2–47.4	47.0–46.8	47.2–47.4	47.4	47.2–47.4

Fig. 1 gives the graphical representations of these numbers; from which it will be seen that the activity for small doses of serum, whether the same individual or different animals of the same species be studied, represents a more or less definite degree of activity—there being but little separation between the individual curves in this region. For larger doses, the curves tend to separate, suggestive perhaps that the enzyme content of the blood is not absolutely fixed, but that it varies at times within certain limits. For the individual, however, the enzyme content of the blood does appear to be remarkably fixed. This is well brought out in the case of the dog 4-P (Fig. 1), the activity curves of which relate to two specimens collected at an interval of 1 week, the two curves being practically identical. The same thing is also seen, though perhaps to a less marked degree, in connection with the dogs 2-P and 3-P. In regard to 2-P, an interval of 1 week separated the specimens 1 and 2, and an interval of 2 weeks the specimens 2 and 3; while for 3-P an interval of 44 days separated the specimens 1 and 2.

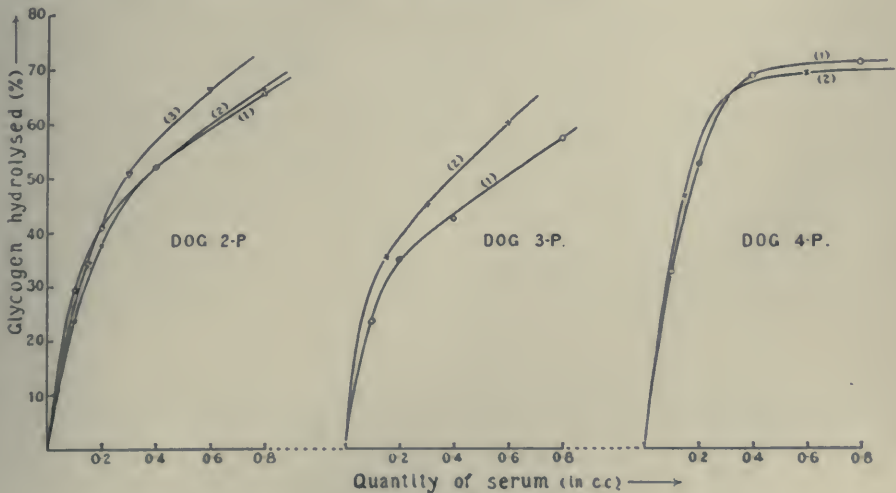


Fig. 1.

Horse. The figures with two different specimens of horse serum, both utilised 4 days after collection are set out in Table II.

Table II.

Dose of serum employed	Glycogen hydrolysed %	
	1	2
0.4	—	1.2
0.8	1.8	2.6
Temp. of exp.	47.4–47.2	46.0–45.9

On graphical representation these figures give the curve represented in Fig. 2, which proves the glycogenolytic power of horse's serum to be extremely feeble.

Ox. Only one specimen of ox serum was examined. This was used 2 days after collection, the temperature during the action being $45.5-45.7^{\circ}$. The results are shown in the curve in Fig. 2.

Pig. One specimen only of pig's serum was examined, the serum at the time of testing being 15 days old; temperature 46° . See the corresponding curve in Fig. 2.

Sheep. A specimen of sheep's serum, shed 26 days, and preserved sterile in presence of toluene, was tested for glycogenolytic power at a temperature of 46.5° . See the curve in Fig. 2.

BLOOD MALTASE NEGATIVE GROUP.

Under this heading, sera from the cat, the monkey, the rabbit, and man were investigated.

Cat. Only one specimen was examined, the serum used being 4 days old; temperature $46.4-44.5^{\circ}$. See Fig. 2.

Monkey. This examination relates to a specimen of blood obtained from a *Macacus cynomolgus*. The serum was fresh when used, that is to say, as soon as clotting began the serum was at once separated from the clot by centrifugation, and the activity experiment then put up. The specimen was without any action on maltose in doses of 0.2 and 0.4 cc. in an action of 16 hours' duration at 46° , thereby proving, as was to be anticipated, that the monkey belongs to the blood maltase negative group of mammals. See Fig. 2.

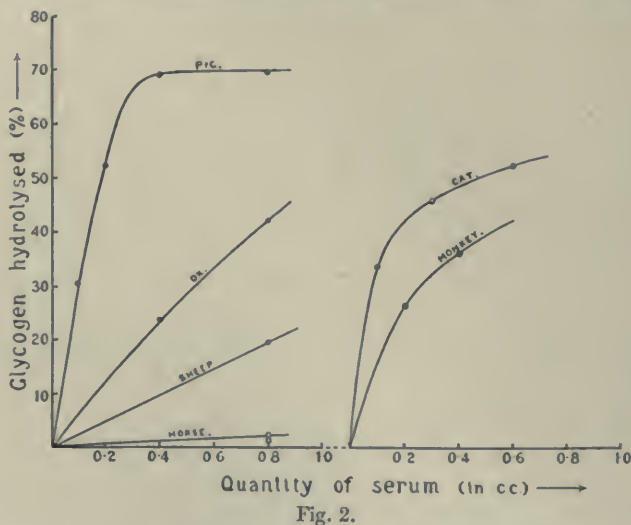


Fig. 2.

Rabbit. Four specimens of rabbit's sera were examined. Blood was collected from 2 rabbits at a time, the blood being "pooled," in connection with specimens 1, 2 and 3, so as to obtain sufficient serum for these and other experiments undertaken with it. The 4 specimens, therefore, correspond in all to 7 rabbits. Table III gives the numbers obtained, and the respective curves are indicated in Fig. 3.

Table III.

Dose of serum employed	Glycogen hydrolysed %			
	1	2	3	4
0.1	8.0	—	—	14.4
0.2	16.9	—	—	—
0.3	—	—	24.4	—
0.4	—	31.0	—	—
0.5	33.6	—	—	37.5
0.8	—	41.0	—	—
1.0	—	—	—	55.0
Age of serum	1 day	1 day	4 days	Fresh
Temp. of exp. °C.	47	46-47	47	46.0-47.4

Man. The activity of normal human serum in glycogenolytic power was tested on three occasions as follows: (1) with a single specimen of serum; (2) with a "pooled" specimen of seven negative Wasserman sera; and (3) with a "pooled" specimen of eight negative Wasserman sera. The figures obtained are given in Table IV.

Table IV.

Dose of serum employed	Glycogen hydrolysed %		
	1	2	3
0.4	4.4	8.0	15.5
0.8	20.8	20.8	25.8
Age of specimen	1 day	6 days	3 days
Temp. of exp. °C.	47.2	47.2	45.6-45.7

When these numbers are represented graphically, the two curves of the right-hand portion of Fig. 3 are obtained. There is the suggestion of a period of lag portrayed by the curve relating to specimens 1 and 2. Whether such findings are accidental, or real, will require further investigation to settle.

DISCUSSION OF RESULTS.

From a consideration of Fig. 1, and the first section of Fig. 2, it will be seen that positive blood maltase mammals, at least the limited number studied by us, when classified in terms of decreasing glycogenolytic activity of their blood give rise to a series, as follows: pig, dog, ox, sheep, horse.

This series presents a slightly different arrangement to that of the corresponding series representing decreasing activity in *maltase*, the latter, under the same experimental conditions being: pig, dog, goat, sheep, horse and ox [Compton, 1921]. A noteworthy point of contrast here, which merits attention, is the comparative richness of the serum of the horse in *maltase*, as compared with its extremely feeble *glycogenase* content. Perhaps, indeed, it would be more in accordance with the real facts, in this connection, to speak of glycogenolytic power; because, however feeble this may be, increased activity can always be brought out by suitable p_{H} alterations of the medium.

Do the foregoing series of sera, of diminishing enzyme content, correspond to any other known properties of sera? Among possible properties one thinks of toxicity. It is a well-known fact that the blood of one animal when transfused into an animal of a different species, is often very toxic: a toxicity which in

the first instance was attributed to the corpuscles. But it has been proved, to quote Richet [1921], that "the real toxic element is the serum itself." It may then be asked, if the serum of one animal contains substances which are toxic for an animal of another species, may not the differing enzyme content of sera play a rôle in this phenomenon? As a matter of fact the toxicity of various sera for man, on intravenous or subcutaneous injection, is given by Richet [1921], in terms of diminishing toxicity, as: dog, goat, ass, horse. Horse serum, then, is the least toxic of mammalian sera for man; and we have shown that, of all the sera studied by us, it possesses the feeblest glycogenolytic power.

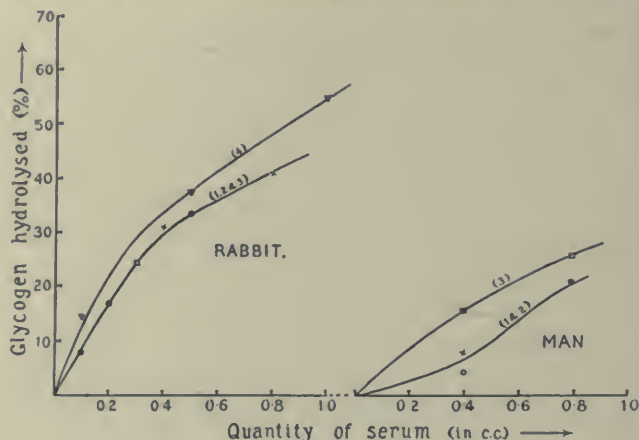


Fig. 3.

But, this conception, of serum toxicity being a function of enzyme content, will be found to rest already on solid ground, if examined from quite a different aspect: the action of heat upon the toxicity of serum. In the case of the most toxic sera known, namely those of the eel—whose serum kills in the dose of $\frac{1}{20}$ th of a cc. per kilogram—the lamprey, certain vipers and cobras, whose serums are almost as toxic as their venoms, Richet states that when heated to 58° , or even lower, the toxic properties of such sera are mostly abolished; so much is this so, that the use of heated sera to-day tends to become general. The progressively destructive action of heat on any solution of enzymes hardly requires being insisted upon.

When the mammals of the negative blood maltase group are studied, it is seen from Fig. 2 (right-hand section) and Fig. 3 that they arrange themselves, in terms of decreasing glycogenolytic activity of their blood, as follows: cat, monkey, rabbit, man.

It is of some interest that man, in this group, should be lowest in the scale of blood glycogenolytic function. Of course that may simply be accidental, and further studies may reveal negative blood maltase mammals of still lower glycogenolytic power. But if not, then it points to the possible fact—allowing for exceptions, like the rabbit above—that loss of glycogen hydrolysing power of blood occurs as we ascend in the mammalian scale.

I would mention here, in passing, that Twort and Archer [1923] have found a practically identical arrangement for animals of our negative blood maltase group, so far as the content of their blood in *lipase* (monobutyrynase) is concerned. In terms of decreasing *monobutyrynase* content the negative blood maltase mammals studied by these workers arrange themselves in the following series: guinea-pig, cat, rabbit, man.

But it may be asked to what purpose are enzymes met with in blood? Some authorities, like Carlson and Luckhardt [1908] consider that they serve no essential end in the body processes, that they are mere "discards" of the tissues on the road to destruction or elimination, and that such differences as are noticeable, for instance in the amylase of blood, at different times appear to be of an accidental nature; or again, as stated by Moeckel and Rost [1910], that they play no rôle in the economy, that oscillations in the amylase of blood are merely expressive of variations in the digestive processes in some organ of the body.

I find it difficult to subscribe to the views thus expressed, that blood enzymes play no rôle in the economy, or that they are simply waste products on their way to elimination and destruction. That these enzymes in the blood are functionally active, and that they are active to different—but more or less definite—degrees for different mammalian species, is at least suggestive that they are present in the blood for their own specific purposes. As shown particularly for the individual dog, successive examinations, under as nearly as possible constant experimental conditions, reveal a remarkable constancy of enzymic activity. For the individual animal, the glycogenolytic activity of the serum gives one the impression of bearing the stamp, so to speak, of the individual; the activity curve is, in this intimate way, perhaps more characteristic of the individual than it is even of the species. In other words, the individual stands portrayed in the enzymic activities of his blood.

It is a pleasure here to express my special indebtedness to M. Albert Frouin who kindly supplied me with the specimens of dog's blood for the investigation, also to Dr C. Levaditi who furnished me with the blood of the monkey; while my thanks are due to Mlle L. Castlenau for help with some of the sugar estimations.

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LXVII. THE ACID NATURE OF OXYHAEMOGLOBIN.

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(Received June 22nd, 1923.)

THE direct demonstration that oxygenated blood is more acid than reduced blood is a difficult experimental feat, owing to the small change of c_H produced in blood by saturation with oxygen. Indeed Bayliss [1923] throws some doubt on the phenomenon, saying that "the chief evidence rests on measurements with the hydrogen electrode and the differences found are within the errors said by Sørensen to be inherent in the method when proteins are present." As a matter of fact the difference found by Parsons [1917] was indeed small, but perfectly definite, and the obvious accuracy and care with which his observations were made scarcely merit the criticism that they lie within the limits of error of the method. The phenomenon is not so unimportant as Parsons' small observed difference might lead one casually to suppose; the very efficient buffers present in the blood naturally lower, to a considerable degree, the rise of c_H due to the production of a small quantity of a stronger acid, and it is not fair to regard the small observed difference of c_H as the chief evidence for the phenomenon. The known effect of acid upon the oxygen-dissociation curve makes it *inevitable* thermodynamically that there should be a corresponding action of oxygen upon the c_H of blood: and the known effect of CO_2 on the same dissociation curve makes it similarly inevitable that there should be a corresponding action of oxygen upon the combination of CO_2 . The effects of CO_2 on the dissociation curve are, as shown by Barcroft and Murray [1923], entirely due to the change of c_H produced by the CO_2 : so that, conversely, the effects of oxygen on the CO_2 -combination are entirely due to acid liberation effected by oxygenation. Hence we have three well-known effects, those of acid and of CO_2 on the oxygen dissociation curve, and that of oxygen on the CO_2 combination, all of which—as facts—are entirely beyond dispute, and each of which is a certain proof of the enhanced acidic properties of haemoglobin on combination with oxygen.

According to Henderson [1908, 1909, 1920], Hasselbalch [1916], Parsons [1919], van Slyke [1921], and many other workers, the whole of the carbon dioxide carried in blood, otherwise than in solution as CO_2 , is there as bicarbonate ions. Hence the effect observed by Christiansen, Douglas and Haldane [1914], viz. the reduction in the combined CO_2 resulting from oxygenation, must be

attributed to the formation of some fixed acid about as strong as CO_2 : the diminished CO_2 capacity is then due to the combination of the available alkali with this other acid. In Fig. 1 the original observations of Christiansen, Douglas and Haldane, are shown, and the upper line through the observations on the reduced blood of J. S. H. is their curve. Let us assume that the combination with oxygen has liberated fixed acid, comparable in strength with CO_2 , in amount equivalent to AB (see Fig. 1). Join A to the origin O , and draw BC horizontally to meet OA in C . Then C in the diagram represents a point at the same c_{H} as A , since the ratio $p \cdot \text{CO}_2/v \cdot \text{CO}_2$ is the same both for A and C , and it is known that in blood—over a fairly wide range of CO_2 pressures— c_{H}

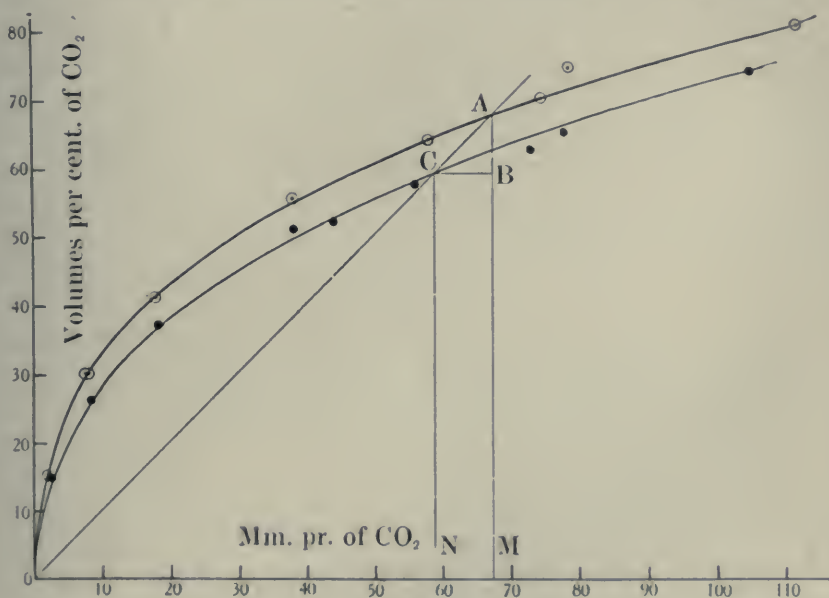


Fig. 1

is proportional to $p \cdot \text{CO}_2/v \cdot \text{CO}_2$. Hence at C any acid in the blood is dissociated to the same degree as at A , so that the same amount of alkali is available at C as at A to combine with added acid or with CO_2 . Thus C is a point on the dissociation curve of the blood after a small amount of fixed acid AB has been added to it. By shifting A along the upper curve, and repeating the construction, the whole of the lower curve can be reproduced. In Fig. 1, AB is taken as 8.4 cc. of CO_2 per 100 cc. of blood, and it is seen that the lower curve drawn in this way fits the observed points with complete satisfaction. Thus the effect of oxygen upon the CO_2 dissociation curve of the blood of J. S. H. may be expressed by the statement that the change from complete reduction to full oxygenation has simply liberated fixed acid, of strength comparable with CO_2 , in amount equivalent (as acid) to 8.4 cc. of CO_2 .

According to the theory put forward and tested by Brown and the present writer [1923] the combination of oxygen with haemoglobin, proceeding accord-

ing to the scheme $\text{H(Hb)}_n + n\text{O}_2 \rightleftharpoons \text{H(HbO}_2)_n$ leads to the formation of the stronger acid oxyhaemoglobin which dissociates according to the scheme, $\text{H(HbO}_2)_n \rightleftharpoons \text{H}^+ + (\text{HbO}_2)'_n$. From experimental data provided by Barcroft and Murray [1922] the acid dissociation constant of oxyhaemoglobin was calculated and found to be about equal to that of H_2CO_3 . According to this theory the stronger acid formed, viz. one molecule of $\text{H(HbO}_2)_n$, which like CO_2 must be almost completely dissociated at the c_{H} of blood, is equivalent in amount to $1/n$ th of the oxygen taken up. This is simply tested. The most probable value of n for blood was found by Brown and Hill, from other considerations, to be about 2.2. Assuming the blood of J. S. H. to have held the normal amount of 18.5 vols. of O_2 %, $1/n$ th of this is 8.4 cc. This is precisely the amount by which it is found necessary to lower (at constant c_{H}) the curve for reduced blood, in order to make it fit the observations on oxygenated blood. No more striking testimony could be demanded to the general adequacy of the theory, than (a) the accuracy with which the curve for oxygenated blood can be deduced from that for reduced blood, and (b) the precise agreement of the single constant (the height AB) necessary for that deduction with the quantity required by the theory.

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LXVIII. NOTE ON THE COLOUR CHANGES IN RATS' FUR PRODUCED BY ALTERATIONS IN DIET.

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(Received June 22nd, 1923.)

THE observations described in this paper arose incidentally in the course of other work and it is thought desirable to publish a brief account, inasmuch as the results may be of interest to others who are working definitely along such lines.

Niemes and Wacker [1922] found that grey-black rats turned brown when fed for a long period on a diet of skim milk and starch; further, the animals regained their original colour when put on a normal diet. Rats fed on starch and full-milk showed no change in colour. These observers suggest that, although the hair-pigment is usually assumed to be derived from protein products (*e.g.* tryptophan, tyrosine), it is possible that other substances are also needed for the manufacture of the pigments, and they put forward the suggestion that the absence of cholesterol from the diet is responsible for the colour change they noted.

Garrod [1909] expresses the view that "the trend of opinion at the present day is towards the view that they (*i.e.* the melanins) are derived from proteins in general, perhaps by the action of a tyrosinase, and that the natural melanins are allied to the melanoid substances which are formed during the hydrolysis of proteins."

The recent work of Saccardi [1922, 1, 2] suggests a pyrrole ring structure for melanins with the possibility of adrenaline as a precursor.

The experiments to be described here indicate a relation between the dietary protein and pigment formation. During the summer months of 1920 some control pied-rats fed on bread and whole milk, began to lose their brown-black colour and became a grey-fawn. About three weeks after the first difference was noticed, the animals had no black hairs at all, and were entirely white and a grey-fawn. It was hot weather at the time and hence it was thought that some preservative in the milk might be responsible for the change in colour. Accordingly other experiments were started using milk from two separate sources, and in 2-3 weeks all the rats showed some change in colour and eventually all the animals became light-coloured. They remained thus for a time, but eventually their hair darkened again, although it was never really black.

Similar results were obtained again in the following summer, but no change of colour could be produced in the winter when the animals were growing more slowly. In 1922 this change of coat colour was obtained in 60 growing rats, some fed on bread and whole milk alone, and others on bread and milk supplemented with kitchen scraps (largely vegetable). Other experimental animals on a high protein diet had jet black coats and showed no variation in colour; therefore it seemed that addition of extra protein might lead to a darkening of the coat.

Twenty-four of the light-coloured animals were divided into two groups; one lot were kept on bread and milk (30 g. bread to 100 cc. milk), and the other half had bread and milk and caseinogen (30 g. bread, 100 cc. milk, 9 g. "food casein" [see Hartwell, 1922]). The latter showed a distinct darkening of their coats at the end of the first week; the coloured patches began to darken at the edges and the darkening spread inwards until at the end of the third week the animals were black and white. The jet blackness of the black was noticeable.

Another series of light-coloured animals were put on a high protein diet consisting of 15 g. bread, 6 g. "food casein," 1.5 g. butter, 0.7 g. salt mixture [Hartwell, 1922], 50 cc. of 3 % marmite. These showed changes exactly similar to those observed in the rats fed on bread and milk and caseinogen. In both cases the rats' coats became black, when the animals were fed on large quantities of protein rich in tyrosine and tryptophan. These experiments, therefore, support the view that the melanins are formed from tyrosine and tryptophan.

Other points noted were:

1. The fur is very thick when the animal is fed on a high protein diet.
2. Adult rats rarely show any change in coat colour, but a few instances have been noted—for example, when the stock animals were fed on kitchen scraps poor in protein, a few animals became lighter in colour. (Just over 1000 litters have been experimented with and only one suckling rat had a light coat, which was noticed about the eighteenth day of suckling.)
3. Microscopically the light-coloured hairs appear similar to the dark ones, except that the pigment is much lighter in shade.

I wish to thank Prof. V. H. Mottram for his help in this work, the cost of which was defrayed by a grant from the Medical Research Council.

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LXIX. A HEAT-STABLE CATALYST IN ANIMAL TISSUES WHICH DESTROYS THE IMINAZOLE RING AND UNMASKS AMINO GROUPS.

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(Received June 21st, 1923.)

AN account of the catalytic destruction of carnosine *in vitro* at 100° by a substance present in skeletal muscle and liver has been previously published [Clifford, 1922]. These experiments showed a progressive loss of the iminazole ring when a solution containing carnosine was kept with skeletal muscle or liver over a period of 3-4 weeks. The amount of iminazole was estimated by a colorimetric method described previously [Clifford, 1921, 1], depending on the coupling of the histidine molecule of carnosine with diazo-reagent. The loss of red colour could be accounted for in two ways. The ring substance might have been joined to others in a more complicated manner, similar to that of the histidine in proteins, and thus be unable to couple with the reagent. Another and more probable solution was that the catalyst actually broke up the ring into some simpler substance. In the latter case it was possible that the nitrogen of the ring would be converted to amino nitrogen and then the increase of amino nitrogen could be estimated by van Slyke's method. Accordingly experiments were carried out in which parallel estimations of diazotisable iminazole and amino nitrogen were made.

Iminazole decrease and Amino N increase in Beef and Water at 100°.

3-5 g. of finely minced beef were placed in test-tubes with about 15 cc. water and the tubes were plugged with cotton-wool and left in a constantly boiling water-bath for 17-24 days. Water was added to the test-tubes when necessary to make up for that lost by evaporation.

At intervals a tube was removed, about 30 cc. water and 5 cc. of 20 % metaphosphoric acid added and the whole made up to 50 cc. and left to stand 12-24 hours. After filtration part of the protein-free solution was tested colorimetrically for carnosine [Clifford, 1921, 1] and van Slyke estimations for amino acids were performed on the other portion, the micro-form of the apparatus being used.

The results are shown in Fig. 1, Curves 32 and 41.

From these results it can be seen that in both cases a three-stepped curve was obtained but in opposite directions, *i.e.* a fall of iminazole was accompanied by a rise of amino acid in the protein-free extract. The rise of amino N is, however, above that which could come from carnosine in the solution and

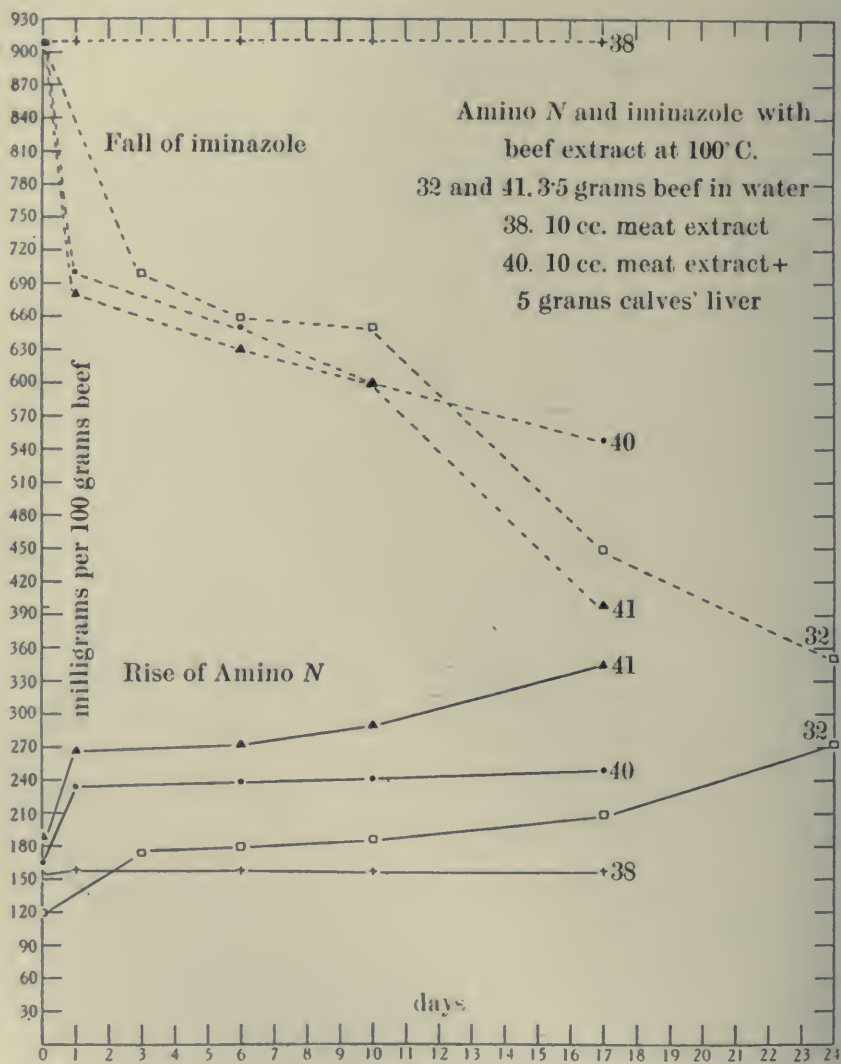


Fig. 1.

thus must come from a hydrolysis of the beef protein itself, or from some nitrogen-containing extractives. These substances are affected in a similar way to the iminazole ring of carnosine and also give a rise of amino N in the same three-stepped manner.

That there are considerable amounts of other soluble extractives giving amino N can be seen from the first figures of Expt. 32 where with meat extract containing carnosine to the extent of 910 mg. per 100 g. meat, 119 mg. amino N per 100 g. were present in place of 52 mg. if the whole came from carnosine. In the case of Expt. 41 again there are 187 mg. per 100 g. instead of 48 mg. if due entirely to carnosine.

The fall of carnosine and rise of amino N is against the findings of Baumann and Ingvaldsen [1918] who found musele and liver extract to be without any hydrolytic action on carnosine. Their experiments, however, assuming enzyme action, were carried out at 40° for a period of 24 hours only.

In one of the author's experiments in which meat and water were sterilised by boiling for 30 minutes and then left at 37° no change in carnosine content was observed in 13 days, so it is unlikely that at 40° any change should take place in 24 hours.

No rise in amino N with Beef Extract.

It was thought possible from the above experiment that the rise in amino N might be due to hydrolysis of soluble protein in the extract, though seven previous experiments [Clifford, 1922] showed that in an extract kept for 13-16 days no change in carnosine content occurs.

Therefore 10 cc. of an extract made by boiling 60 g. of beef in two separate portions of 150 cc. water were placed in each of a series of test-tubes, plugged and left at 100°.

Estimations of carnosine and amino N were carried out on these at intervals. The carnosine remained constant, and the amino N showed an insignificant rise of 4 mg. per 100 g. meat extracted, whilst in the previous experiment the rise was 158 mg. per 100 g. (Fig. 1, 38.)

It is possible that the very small protein content of these tubes compared with that of those in which 5 g. of meat including its fibrin remained in a small bulk of water could account for the difference. This is improbable but experiments with strong protein solution in water at 100° are in progress to ascertain if a large rise of amino N in a three-stepped curve will result.

The heat-stable catalyst responsible for the results obtained above is not confined to musele tissue as the following experiments will show.

Rise of amino N and Decrease of Iminazole with Liver in Meat Extract.

To separate test-tubes, each containing 10 cc. of beef extract made by boiling minced beef with water and filtering, 5 g. of calf liver were added. These tubes were plugged and placed in the water-bath and treated in the same way as the beef and water tubes. Again there was a rise in amino N and a fall in carnosine content, both with the usual curve. The rise of amino N once more was greater than could be accounted for by the conversion of the diazotisable N to amino N (Fig. 1, 40), indicating the presence of other substances than carnosine, which are split by the same agent in a similar way to carnosine.

The question then arose as to whether the catalyst would destroy iminazole compounds other than carnosine. Histidine was therefore used in place of muscle extract and the following experiments were carried out.

Effect of Cod Muscle on Histidine Solution.

Cod muscle has previously been shown to be free from any iminazole substance capable of coupling with diazo-reagent [Clifford, 1921, 2]. It has also been shown to contain a catalyst which destroys carnosine [Clifford, 1922], and was therefore used to determine if the catalyst could act on a simpler iminazole compound.

Separate tubes, containing 10 cc. of 0.05 % histidine monohydrochloride solution, were taken and 5 g. of minced cod muscle were added. These tubes were treated in the same way as those with beef and water. Again the iminazole which could be diazotised was destroyed, and there was a rise of amino N. This was greater than could have come from the histidine and therefore must have come from the cod. At the end of 21 days there was a loss of histidine which could account for a rise of 9.9 mg. of nitrogen if all the ring N were converted to NH_2 , whilst the actual rise was 43 mg.

However, the shape of the curves was the same as with carnosine in meat extract, both with regard to iminazole fall and to amino N rise (Fig. 2, 35). From this it would appear that the catalyst will attack histidine in the same way that it will attack β -alanyl-histidine and may be capable of splitting any iminazole ring.

Effect of Liver on Histidine Solution.

The experiment was carried out as for cod, except that 5 g. of minced calf's liver was used in place of cod muscle, again with the idea of introducing a tissue containing the catalyst which at the same time was devoid of free iminazole. The result was similar to that obtained with cod, namely, a fall of iminazole giving a red colour when diazotised, and a large rise of amino N (Fig. 2, 36).

The rise of amino N here is 42 mg. whilst the most which could have come from the destroyed histidine is 8 mg.

Effect of Washed Meat on Histidine Solution.

It has previously been shown [Clifford, 1922] that meat which has been minced and washed in running water for 24 hours still contains the catalyst, though obviously devoid of all water-soluble substances. Accordingly 5 g. of minced beef washed for 24 hours were added to another series of tubes containing 10 cc. of 0.05 % histidine monohydrochloride solution. On repeating estimations as for the cod muscle and calf liver series similar results were obtained. There was a still greater rise of amino N, it being 126 mg. at the end of 21 days against a possible 9.2 mg. from histidine nitrogen (Fig. 2, 37).

That the destruction of the histidine ring is not mere hydrolysis at 100° is shown by the following experiment.

Effect of Temperature on Histidine Solution.

Tubes containing 10 cc. of 0.05% histidine monohydrochloride solution were placed in the bath at 100° and used as controls. In 21 days there was no alteration either in iminazole content as estimated colorimetrically or in amino N as estimated by the method of van Slyke.

0-3-5-9-17-21 days; histidine 50 mg.; amino N 3.7 mg. (Fig. 2, 34).

From this experiment it appears that there is no spontaneous splitting of the histidine ring when a weak solution is kept at 100° constantly for 3 weeks.

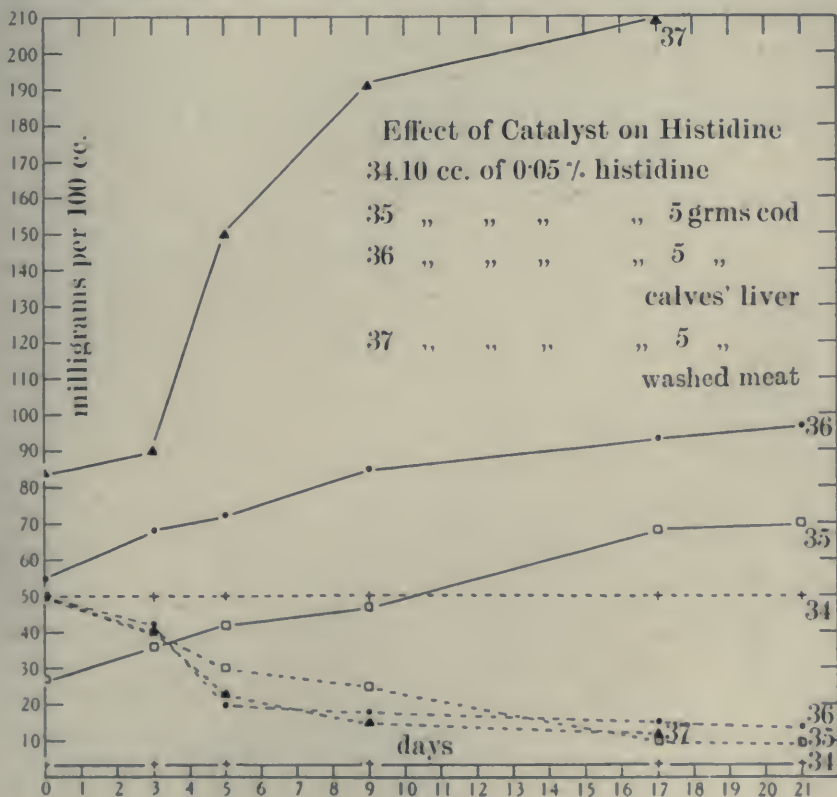


Fig. 2.

DISCUSSION OF RESULTS.

The catalytic agent present in beef and cod muscle and in liver is capable of destroying carnosine in a beef extract, and also of destroying histidine in watery solution. This is shown by a decrease of colour on diazotising histidine solution or muscle extract at intervals after the addition of cod, washed beef or liver, the tubes being kept at 100°. This may indicate the existence of a catalyst capable of splitting any iminazole ring.

The peculiar shape of the curve previously observed in the disappearance of carnosine is maintained with histidine solution. At the same time that there is a fall in iminazole there is a rise in amino N which gives a curve in the opposite direction to the iminazole fall. This suggests an opening of the iminazole ring with the changing of its ring nitrogen to amino N.

The rise of amino N is greater than can be accounted for by the opening of the diazotisable iminazole ring and therefore it must in part come from some other source. The protein of the added substance (muscle or liver) may be hydrolysed with the consequent freeing of amino groups. This is improbable as a watery meat extract which necessarily contains some protein can be kept 17 days with a rise of only 4 mg. per 100 g. extracted meat and the iminazole content remains unchanged. Another objection to the simple hydrolysis of protein as a cause of the rise of amino N is the peculiar shape of the curve obtained. This is the converse of that of loss of diazotisable iminazole and suggests a connection between the two changes. This is shown well in Fig. 3, Expt. 35, where the scale has been enlarged.

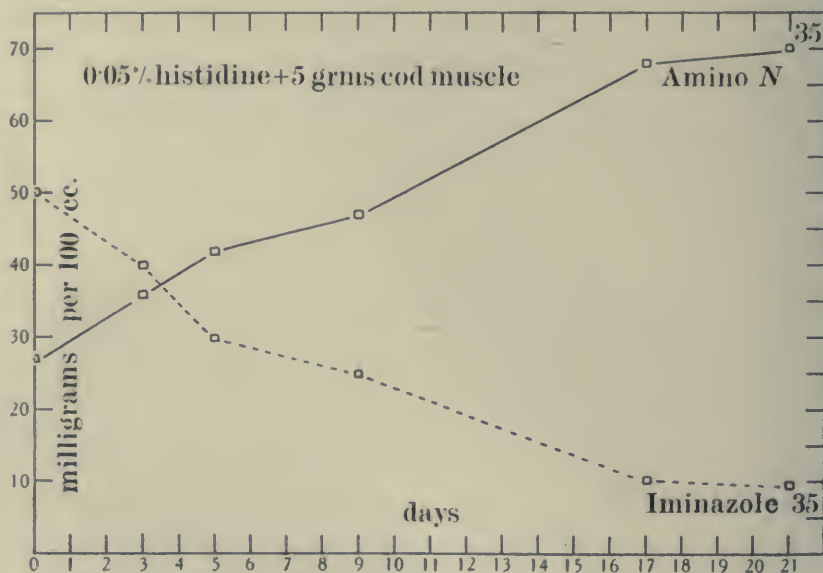


Fig. 3.

The catalyst may be able to attack other iminazole compounds, so that other extractives besides carnosine may give rise to the extra amino N. There is a possibility that purine bodies such as xanthine and hypoxanthine may have their iminazole group split off as urea, and this could account for discrepancies between ingested purine and excreted uric acid in the body. Creatine is, through guanidine, allied to arginine, and also is known to give rise to creatinine spontaneously when boiled. Creatinine shows the iminazole ring formation and therefore is allied to histidine and the purines. The breaking

of the creatinine ring may be possible by the action of the catalyst destroying histidine, when creatine might result. This would give possibilities of a link between arginine and histidine and their interchangeability in the body.

Experiments are in progress in order to enquire into these various possibilities.

Thanks are due to Professor V. H. Mottram for his advice and interest in the work.

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LXX. POLARIMETRIC OBSERVATIONS ON SOLUTIONS OF GLUCOSE SUBJECTED TO CONTACT WITH INTESTINAL MUCOSA OF RABBIT.

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(Received July 4th, 1923.)

IN a paper by Hewitt and Pryde [1920] published in this Journal, three polarimetric experiments with previously equilibrated hypotonic solutions of glucose, which had been in contact with the mucosa of the small intestine of the rabbit for five minutes, are quoted with data which are claimed as indicative of a disturbance of the equilibrium of the solutions as a result of the intestinal contact.

An upward mutarotation of the solution in the polarimeter to the $\alpha - \beta$ equilibrium value is quoted for each of the three cases, the conclusion drawn being that a downward mutarotation had taken place earlier, *i.e.* during the period of contact.

The polarimetric observations were commenced from $3\frac{1}{2}$ to 10 minutes after removal of the solution from the gut, and the mutarotation effect had ceased to be evident from 15 to 25 minutes after removal.

The observers, however, state on pp. 399–400 of their paper that in some experiments these mutarotation effects were “ill-defined,” and in some “completely negative,” but the relative numbers of the experiments yielding the three types of results are not given.

As, so far as we are aware, the positive result of these observers has not been controlled, we have thought it advisable to repeat the experiment. One of us (D. S.) is responsible for the polarimetric and analytical work, and the other (E. W. R.) for the operative procedure.

The observer with the polarimeter took no part in the operation upon the animal, and entered a dark room ten minutes before taking an observation, for the double purpose of attaining a fair degree of dark adaptation of the eye for the earliest readings, and the necessary mental composure.

Since the question as to whether or no any mutarotation occurs in the glucose solution during the early period after its removal from the gut is, in this particular case, of more importance than quantitative values in relation to the concentrations of glucose given by analyses, no attempt at complete chemical removal of the small amounts of protein necessarily added to the solution from the intestinal glands during its sojourn in the gut, was attempted.

In cases such as that before us, we are, moreover, of opinion that chemical treatments may hold an element of danger.

Barring the necessary filtration, the only treatment to which we have ever subjected the solution has been to shake it, previous to filtration with a suspension of aluminium hydroxide. The p_H of the liquid of this suspension was 7.3 by the indicator method, and it was considerably less alkaline, by the same test, than the glucose solution as removed from the gut. The positively charged particles of this suspension are remarkably efficient in clarification, and the filtrate of gut contents so treated may give no "ring" with Tanret's reagent, and in all cases it is perceptible with difficulty.

In many experiments, as evident in the tables, we have purposely omitted the alumina treatment, but though such omission often entailed resort to a more powerful light source than that of sodium flame, it was found to be absolutely without effect on the order of the polarimetric readings, provided that the filtrate was clear enough for good readings.

Procedure. Rabbits, starved 24 hours, anaesthetised by ether, and tracheotomised were employed. About 30 inches (live length) of small intestine, which had been washed free of debris by Ringer-Tyrode solution at 38°, were used, and from 20 to 30 cc. of glucose solution at 38° were run in, distention of the gut being avoided.

No loss of epithelium ever occurred, and, during the contact period, cloths, wrung out in warm saline solution, protected any part of the gut which was not enclosed within the abdominal cavity.

Usually the solution was allowed to lie in contact with the mucosa for five minutes, and if peristalsis was not marked, an occasional gentle manipulation was employed to bring fresh parts of the contents into contact with the epithelium.

Occasionally the solution was kept in the gut for ten minutes, but no difference was noted, so far as mutarotation is concerned, in such instances. The time of removal of the solution from the gut was "clocked," and the clock handed in to the observer in the dark room, as soon as the filtration and filling of the polarimeter tube were completed.

Filtration. Since clarity is a *sine qua non* in a solution, the polarimetry of which is to be reliable, particular attention was devoted to this matter. Clarity of filtrate has, however, in this matter, to be combined with speed of filtration, and the latter condition rules out many otherwise excellent methods. The Balston "filter-hat" used by Hewitt and Pryde is quick, but we found it often at fault in the matter of clarity of filtrate.

We have used a pair of Gooch crucibles, with asbestos mats, built up freshly for each experiment on a disc of filter paper, the two crucibles being in parallel on the suction system, and used contemporaneously.

The number of crucibles in parallel can, of course, be increased, but we found two sufficient for the purpose of getting enough filtrate in a short time. The suction system stood ready with a partial vacuum of about 400 mm. of

mercury, the filter-flasks being clamped off. Once the crucibles were filled with solution from the gut, release of the clamps at once set going the filtration.

In the filter-flasks were set graduated tubes to receive the filtrate, and, by this device, the moment at which enough filtrate had collected to fill a polarimeter tube was known, and no time was lost in unnecessary prolongation of the filtering process.

The polarimeter tube, filled with filtrate, and the clock giving the time at which the original solution left the gut, were at once handed in to the observer in the dark room.

The filtrates, when alumina cream is used, are "glass clear," and the Tanret protein test is negative or only just perceptible. As the glucose analyses were made on the contents of the polarimeter tubes, the slight dilution due to the use of the alumina cream was of no moment in the matter of comparison of direct and calculated readings.

We do not, however, pretend that all laevo-rotatory substances of intestinal origin added to the original glucose solution are removed by this elementary procedure. With good working asbestos mats we often got a first reading on the polarimeter in two minutes after removal of the solution from the gut, but the time which must elapse to collect a sufficient filtrate is necessarily variable, by the "build" of the mat, and the amount of the secretion which has occurred in the gut affecting the viscosity of the solution submitted to filtration. The times will be found in the tables of experiments.

Polarimetry. The instrument was a Schmidt and Haensch three-field of the Lippich type, reading direct to 0.01° , and either one or two decimeter tubes were used. The zero value of the tube (water-filled) was independently taken for each experiment. To preserve, as far as possible, the dark adaptation of the observer's eye the light for scale reading was cut down to the minimum practicable.

Though sodium light served well for the filtrate which had undergone alumina cream clarification, difficulties in sharp reading were often met with when this treatment was omitted. As already noted, we have purposely omitted the alumina treatment in some cases, though it has not in any way affected the results. While a good sample of asbestos mat will give a clear enough filtrate, without any alumina clarification, others may give a filtrate too opalescent for proper observation by sodium light.

As, at the time, we had no arrangement for intense mercury-green light we had in those cases to resort to light-filters, and a 100 C.P. "Pointolite" lamp. The filters used for the light were Wratten's M. screens. $A + B + E$ in conjunction. The abandonment of monochromatic light in such cases, while affecting the comparison of calculated and observed rotations, does not, of course, affect the question under examination, viz. whether mutarotation is or is not to be observed in the solutions shortly after removal from the gut.

Glucose solutions and analytical method. Hewitt and Pryde do not state whether the solutions introduced into the gut were simple aqueous solutions

of glucose or solutions in some type of saline liquid. Most of our experiments were done with simple aqueous solutions; occasionally the glucose was dissolved in Ringer-Tyrode solution, and, in one instance, acid sodium phosphate was added to an equilibrated glucose solution before use, to try the effect of keeping the gut contents on the acid side throughout the experiment.

The concentration of the solution subjected to polarimetry was estimated by the micro-method of Shaffer and Hartmann [1921]. The limited volume of solution available for analysis necessitated the use of a reliable micro-method so that as many individual analyses as possible might be effected for a representative mean value.

As some laevo-rotatory material is, by the nature of the experiment, always present in the mixture of glucose and intestinal secretion subjected to polarimetry, the rotation calculated from the analysis is likely to be greater than that observed. In nine cases out of the thirteen, where observations were made by sodium light, this has been found to be the case. In the experiments where the protein tests were most distinct, this difference between calculated and observed rotations was greatest.

Furthermore, in such cases the opalescence of the solutions was sometimes sufficient to impair the reliability of the polarimetric readings.

An instance, not included in the tables, is given of a series of readings from 4 to 25 minutes after removal of the solution from the gut, in which the "switch-back" character of the readings, due to absence of clarity, makes the observation valueless.

Minutes since re- moval from gut	4	5	6	7	8	9	10	11	12	13	14
Observed dextro- rotation in degrees	·85	·82	·80	·80	·80	·79	·78	·79	·80	·79	·77
	15	16	17	18	19	20	21	22	23	24	25
	·78	·78	·76	·76	·75	·77	·77	·80	·80	·80	·77

The erratic up-and-down variation of the readings in this instance, beyond the limits of the error of reading on a clear tube, is obviously due to the absence of certainty in such a case in adjustment of the field of the instrument. Any results of this type have been rejected.

Effects of alkalisations of contents of polarimeter tube. The addition of a drop of alkali to the contents of the polarimeter tubes, results, with one or two exceptions, in a distinct diminution of the dextro-rotation. We found, invariably, that the greater the amount of protein in the filtrate, the greater the diminution on addition of alkali, and, the clearer the filtrate, the smaller the effect of alkali.

We have also observed that the addition of weak alkali (2 % NaOH) results in no change or in a slight increase. On the other hand, the addition of a drop of strong alkali (40 % NaOH) results in a diminution sometimes of 0·1° or more in a 1-decimeter tube. These filtrates were alkaline enough, from added

Table 1. *Sodium Light Readings.*

Dextrose concentration introduced into gut. Treatment of filtrate etc.	Mean dextro-rotations in degrees at minute intervals after removal from gut. Readings from 2-25 minutes																				Dextro-rotation in degrees		No. of tube		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23		24	25
1 % dextrose in Kinger- Tyrode, Alumina. Asbestos filters. 2 d. tube	—	·79	·78	·77	·79	·79	·81	·81	·82	·78	·79	·78	·79	·80	·78	·78	·79	·79	·79	·79	·81	·79	·79	·79	13
2 % dextrose in .96 % NaH ₂ PO ₄ , Alumina. As- bestos filters. Hypotonic △ = -0.43° C. 2 d. tube	—	·15	·13	·10	·10	·10	·11	·10	·10	·11	·11	·11	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	18
2 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	24
Ditto	—	—	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	·13	25
• Ditto	—	—	—	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	·17	26
Ditto	·98	·99	·97	·96	·98	·97	·98	·98	·98	·97	·98	·98	·99	·98	·97	·97	·98	·98	·98	·98	·98	·98	·98	·98	28
2 % dextrose in water No alumina. Filter hat. 1 d. } tube	·79	·80	·80	·80	·78	·80	·79	·79	·80	·78	·79	·78	·80	·78	·79	·79	·80	·79	·78	·80	·78	·79	·79	·79	11
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	·10	20
Ditto	—	—	·98	·99	·102	·98	·99	·102	·100	·102	·100	·102	·99	·99	·99	·97	·98	·100	·101	·99	·101	·100	·99	·100	27
Ditto	·99	·100	·100	·99	·99	·100	·99	·98	·99	·99	·99	·99	·100	—	—	—	—	—	—	·97	·100	·99	·100	·99	16 (b)
Ditto	—	·107	·108	·108	·107	·108	·107	·107	·107	—	—	—	—	·105	·105	·103	·106	·105	·105	—	—	—	—	—	15 (b)
1.7 % dextrose in water, No alumina. Asbestos filters. 1 d. tube	·84	·83	·83	·81	·80	·82	·82	·83	·83	·84	·84	·82	·82	·81	·82	·84	·83	·83	·84	·82	·83	·82	·82	·83	30
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	·96	·98	·99	·98	·95	·97	·99	·97	·99	·98	·97	·99	·97	·97	·97	·98	·97	·98	·97	·96	·97	·97	·97	32

Table II. Screened "Pointolite" Readings.

Dextrose concentration introduced into gut, Treatment of filtrate etc.	Mean dextro rotations in degrees at minute intervals after removal from gut. Readings from 3-25 minutes																				Dextro-rotations in degrees			No. of tube				
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		(1) After standing one hour	(2) After adding alkali	(3) Recalculated from analyses	% dextrose from analyses
1 % dextrose in Ringer-Tynale. No alumina. Filter hat. 1 d. tube	.24	.23	.23	.23	—	.24	.24	.24	.24	.24	.24	.24	.24	.24	.25	.25	.25	.24	.25	.24	.25	.25	.24	.24	.20	.21	.306	12
1.5 % dextrose in Ringer-Tynale. Alumina. Asbestos filters. 2 d. tube	—	.53	.56	.56	—	—	.59	.58	.55	.55	.55	.55	.55	.55	.55	.55	.55	—	—	.55	.55	—	.55	.57	.56	.60	1.148	14
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	—	—	.78	.79	.78	.78	.78	.76	.76	—	—	—	—	—	—	—	—	—	—	—	—	.76	.78	.67	.74	1.460	16 (a)
Ditto	—	—	—	.63	.62	—	.64	.64	.64	.64	.63	—	—	—	—	—	—	—	—	.64	—	.64	.63	.63	.65	.77	1.458	15 (a)
1 % dextrose in water. No alumina. Asbestos filters. 2 d. tube	.96	.97	.97	.97	.96	.96	.96	.96	.97	.96	.97	.96	.97	.98	.97	.98	.98	.98	.97	.97	.97	.98	.97	.96	.94	1.10	1.048	6
Ditto	1.05	1.07	1.06	1.06	1.06	1.06	1.06	1.06	1.08	1.08	1.08	1.09	1.08	1.08	1.10	1.08	1.09	1.08	1.08	1.08	1.08	1.08	1.08	1.08	.99	1.18	1.124	8
1 % dextrose in water. No alumina. Filter hat. 2 d. tube	.90	.90	.90	.89	.88	.90	.91	—	.88	.89	.87	.87	.87	.89	.89	.89	.91	.89	.88	.88	—	—	.88	.88	.80	.99	.942	9
1.8 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	.69	.69	.70	.69	.68	.70	.70	.69	.69	.71	.69	.69	.68	.69	.69	.69	.69	.69	.69	—	.68	.67	.64	.58	.75	1.42	29
2 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	—	—	—	—	—	1.32	1.30	1.34	1.34	1.25	1.33	1.36	1.34	1.33	1.34	1.34	1.35	1.35	1.35	1.35	1.35	1.34	1.36	1.22	1.44	1.368	3
3 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	.91	.91	.91	.91	.91	.91	.91	.92	.91	.91	.90	.91	.90	.90	.91	.91	.91	.89	—	.91	.91	.90	.91	.86	1.00	.952	4
Ditto	—	.84	.84	.83	.85	.84	—	—	.81	.81	.81	.81	.81	.81	.81	.80	.80	.81	.81	.82	.80	—	.80	.81	.74	.88	.84	5
5 % dextrose in water. No alumina. Gut washed with saline. Asbestos filters 1 d. tube	—	—	—	.78	.78	.78	.77	.78	.78	.78	.78	.79	.78	.78	.79	.79	.79	—	—	—	.79	.79	.78	.79	.72	.87	1.65	22
6 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	.82	.81	.80	.79	.79	.80	.79	.80	.79	.80	.80	.79	.80	.79	.79	.79	.80	.80	.79	.79	.79	.80	.80	.69	.91	1.74	31

v Clogged filter.

intestinal secretion, to turn litmus paper blue before addition of the alkali. The same drop in rotation can be got by adding hydrochloric acid. An example of the effect of weak alkali can be seen in the Expts. 13, Table I, and 15 *a*, Table II, while the others all show the effect of a drop of the strong alkali.

These observations lead us to believe that this drop in rotation is due to the protein forming salts with alkali or acid, these salts being more laevo-rotatory than the natural protein—an observation made, among other workers, by Pauli [1914].

REMARKS.

It is evident that, so far as polarimetric evidence goes, our experiments give no support to the idea that a disturbance of equilibrium associated with the formation of a more reactive type of sugar occurs as a result of contact of a stabilised glucose solution with the mucosa of the small intestine of the rabbit.

It appears to us that, even if some reactive type were formed, it is very unlikely that evidence of its production would be obtained by the method adopted by Hewitt and Pryde.

Not only would one expect the special type of sugar formed to be at once taken up into the blood-stream, if the transformation was a necessary preliminary to absorption, and so to be removed from the solution used for experimental observation, but, even if traces of the special type still remained in the solution withdrawn from the gut, its existence in unstable form is likely to be momentary.

According to Irvine [1923] the gamma types of both glucose and fructose revert *instantaneously* to the stable form even in the presence of 0.1 % HCl.

Aqueous solutions of glucose which have been in contact with the gut wall for 5 minutes are always alkaline, so far as our experience has gone.

To prove the thesis that a reactive type is formed, some method of condensation to a stable derivative would appear to be necessary for, as Irvine remarks, "there is little prospect of detecting these fugitive isomerides by ordinary processes."

To effect such a "fixation" within the organism, preliminary to extraction and investigation, seems to be the problem before the searcher for evidence of the transitory presence of reactive types of sugar in metabolism.

CONCLUSION.

The experiment of Hewitt and Pryde has been repeated thirty-four times. In eight cases the solutions obtained from the gut had to be discarded as not clear enough for good polarimetry.

In the remaining twenty-six experiments in no single instance have we seen any sign of mutarotation in the earlier minutes after removal of the solution from the gut.

The rotation has remained steady within the observer's known error of adjustment of the field of the polarimeter on ordinary stable solutions of glucose in water.

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LXXI. URIC ACID AND ALLANTOIN EXCRETION AMONG OFFSPRING OF DALMATIAN HYBRIDS.

BY THE LATE HERBERT ONSLOW.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Council.

IN a previous communication [Onslow, 1923] evidence was given for the fact that hybrids, from a cross between the Dalmatian hound and a terrier, excrete uric acid and allantoin in relatively the same proportion as in the normal dog. The appearance of the two hybrids, a dog and a bitch, was there described, the former being without spots, the latter only very slightly spotted.

In the late autumn of 1922, the hybrid dog and bitch were mated. Six puppies were born on December 26th, 1922. With the purine metabolism of these, the present communication is concerned. Photographs of five of the puppies, *A*, *B*, *C*, *D* and *E*, at the age of about 5 months, are shown in Plate VIII. Two of the puppies were reared only with great difficulty¹, and one, a male, died at the age of 8 weeks. Three of the puppies developed spots, namely, *D*, *E* and the one that died. The spots did not begin to appear until some 3 or 4 weeks after birth.

The bitch *A* (Plate VIII, Fig. 1) resembles a terrier; the ears are yellow and she has a yellow patch round one eye with a slight development of black pigment. The bitch *B* (Plate VIII, Fig. 2) also resembles a terrier; she has brindled ears and patches round the eyes. The bitch *C* (Plate VIII, Fig. 3) is without pigment except for a small black spot or two on the nose and a small yellow patch on the ear. The bitch *D* (Plate VIII, Fig. 4) resembles a true Dalmatian, being spotted more or less regularly over the body and more heavily on the ears. The bitch *E* (Plate VIII, Fig. 5) is a good deal spotted, but not so regularly as *D* and has also black ears, black eye patches, a black patch on the right fore flank and one on the tail.

The puppies were trained for the dog cage early, and the method of procedure was as described in the previous paper, except that the duration of time in the cage did not exceed 12 hours. In the case of all the animals, two preliminary estimations of uric acid were performed before the estimations of both uric acid and allantoin were made simultaneously. The diet was chiefly puppy biscuit and was much less varied than in the case of the parents previously described.

¹ The estimations in the present (as in the previous) paper were carried out by Mr John Lowndes, assistant to the late Mr Onslow. The success of the research is entirely due to Mr Lowndes' skilful and unflinching care for the puppies. (M.W.O.)

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Fig. 1. Bitch *A*.



Fig. 2. Bitch *B*.



Fig. 3. Bitch *C*.



Fig. 4. Bitch *D*.



Fig. 5. Bitch *E*.

The results are recorded in Table I. *Bitch A* was 12 weeks and 6 days old at the date of the first experiment, and 17 weeks and 1 day at the date of the last experiment. At the beginning of the experiments she weighed 3.2 kilos. (7 lbs.), and at the end, 4.5 kilos. (10 lbs.). The duration of time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3 and 4, 12 hours.

Table I.

Bitch A.

Date of experiment 1923	Vol. of urine cc.	Sp. gr. of urino	In whole volume of urine			Uricolytic index	Percentage of total N as	
			Total N g.	Uric acid N g.	Allantoin N g.		Uric acid N	Allantoin N
1. March 26	83 (100)*	—	0.74774†	0.00165†	—†	—	0.22	—
2. April 6	53 (100)	—	0.76600	0.00094	—	—	0.12	—
3. „ 20	132 (200)	1.020	1.47454	0.00424	0.31132	99	0.29	21.13
4. „ 25	120 (200)	1.025	1.53314	0.00232	0.32910	99	0.16	21.46
Average for Exps. 3 and 4 only:						99	0.22	21.29

Bitch B.

1. March 27	64 (100)	—	0.58136	0.00173	—	—	0.30	—
2. April 9	92 (100)	—	0.78020	0.00180	—	—	0.24	—
3. „ 16	142 (200)	1.020	1.39249	0.00472	0.28803	98	0.34	20.69
4. May 2	172 (200)	1.021	2.16606	0.00692	0.45723	98	0.32	21.11
Average for Exps. 3 and 4 only:						98	0.33	20.90

Bitch C.

1. March 20	30 (50)	—	0.33227	0.00233	—	—	0.70	—
2. „ 28	64 (100)	—	0.72948	0.00282	—	—	0.34	—
3. April 11	75 (150)	—	1.15856	0.00540	0.22046	98	0.47	19.03
4. „ 30	206 (250)	1.016	1.41829	0.00699	0.29417	98	0.43	20.74
5. May 11	210 (250)	1.012	1.50620	0.00236	0.26487	99	0.16	17.58
Average for Exps. 3 and 4 only:						98	0.45	19.88

Bitch D.

1. March 21	32 (50)	—	0.46416	0.00264	—	—	0.57	—
2. April 4	43 (50)	—	0.61734	0.00237	—	—	0.37	—
3. „ 23	170 (200)	1.015	1.20496	0.00377	0.25879	99	0.31	21.48
4. May 9	145 (200)	1.021	1.91992	0.00503	0.38690	99	0.26	20.15
5. „ 15	65 (200)	1.066	0.77369	0.00094	0.08883	99	0.12	11.48
Average for Exps. 3 and 4 only:						99	0.28	20.81

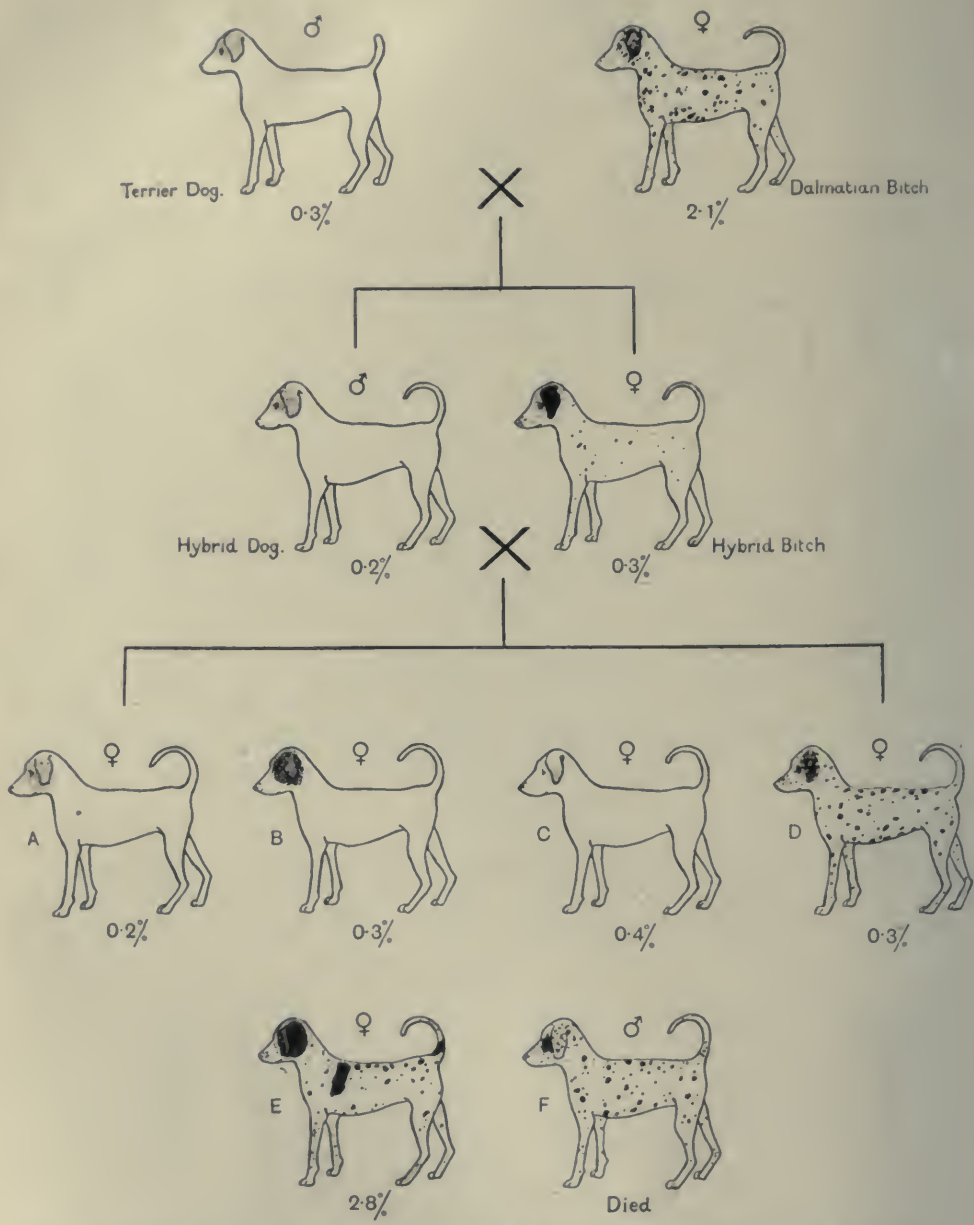
Bitch E.

1. March 23	72 (100)	—	0.29122	0.01221	—	—	4.19	—
2. April 5	85 (85)	—	0.56142	0.01337	—	—	2.38	—
3. „ 18	120 (200)	1.021	1.38603	0.04246	0.23534	85	3.06	16.97
4. May 7	160 (200)	1.020	1.58062	0.03931	0.27030	87	2.49	17.12
5. „ 14	185 (200)	1.020	1.90821	0.04938	0.28808	85	2.59	15.10
6. „ 16	160 (200)	1.025	3.07628	0.05189	0.25878	83	1.69	8.41
Average for Exps. 3 and 4 only:						86	2.77	17.04

* Figures in brackets denote volume of first dilution.

† Unfortunately four figures only were given in these columns in the previous paper [Onslow, 1923]. The percentages, however, were calculated on six figures and are therefore correct and strictly comparable with those given in the present paper.

Bitch B was 13 weeks old at the date of the first experiment, and 18 weeks and 1 day at the date of the last experiment. At the beginning she weighed 2.7 kilos. (6 lbs.) and at the end 4.5 kilos. (10 lbs.). The duration of time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3 and 4, 12 hours.



Scheme showing the inheritance of the Dalmatian characters in the first and second generations.
The numbers denote percentages of uric acid nitrogen of total nitrogen.

Bitch C was 12 weeks old at the date of the first experiment, and 19 weeks and 3 days at the date of the last experiment. At the beginning of the experiments she weighed 4.1 kilos. (9 lbs.); at the end 6.6 kilos. (14½ lbs.). The time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3, 4 and 5, 12 hours.

During Exp. 5 the animal refused to eat; hence the results are not strictly comparable with the previous experiments and will be considered later.

Bitch D was 12 weeks and 1 day old at the date of the first experiment, and 20 weeks at the date of the last experiment. At the beginning of the experiments she weighed 2.7 kilos. (6 lbs.), and at the end 4.5 kilos. (10 lbs.). The time in the cage for Exp. 1 was 8 hours; for Exp. 2, 7 hours; for Exps. 3 and 4, 12 hours and for Exp. 5, 13½ hours.

Again, Exp. 5 was not comparable to the others, as the animal refused to eat while in the cage.

Bitch E was 12 weeks and 3 days old at the beginning of the experiments, 20 weeks and 1 day at the end. She weighed 3.6 kilos. (8 lbs.) at the beginning of the experiments and 6.3 kilos. (14 lbs.) at the end. The time in the cage for Exps. 1 and 2 was 8 hours and for Exps. 3, 4, 5 and 6, 12 hours. Exp. 6 took place after a diet of water biscuit for 24 hours.

A practice was made of feeding the animals while in the cage. Towards the end of the experimental period, three, namely *C*, *D* and *E* refused to eat. It will be seen, from Exps. *C* 5, *D* 5 and *E* 5 that starvation reduced the percentage of allantoin. Further, for Exps. 1 and 2 in each case the animals were very young. Hence the average of Exps. 3 and 4 is considered the most representative for the normal animal in each case. They are recapitulated as follows:

	Percentage of total N as	
	Uric acid N	Allantoin N
<i>A</i>	0.22	21.29
<i>B</i>	0.33	20.90
<i>C</i>	0.45	19.88
<i>D</i>	0.28	20.81
<i>E</i>	2.77	17.04

In regard to the allantoin excretion, the amounts excreted (except in the starvation experiments) were much more constant than in the case of the parents [Onslow, 1923]. This is probably due to the fact that the diet was more constant, the animals being fed almost entirely on biscuit. Starvation, either self-imposed or designed, as in Exp. *E* 6, reduces the allantoin percentage. At the same time it also reduces the uric acid percentage but does not appear to reach the condition, described by Benedict [1916], in which the animal may excrete a higher percentage of uric acid than of allantoin.

From the urine of the bitch *E*, uric acid crystals separated on adding 10 % hydrochloric acid and allowing to stand.

In regard to the uric acid excretion, it is clear, from the results in the second generation, that there has been a segregation of the character involving the power to destroy this substance from the character involving lack of this

power. Apart from this outstanding fact, it is difficult to postulate the exact course of inheritance from the data given by this one family alone. The results, however, suggest that, in main outline, the grandfather terrier (probably of mixed breed) carried a dominant factor for destruction of uric acid; also a factor for the inhibition of spotting which is not always entirely dominant in the hybrid. If it is assumed that these two factors segregate independently, then it is a simple two-factor Mendelian case (though there are, without doubt, subsidiary complications). Sixteen individuals would then, theoretically, be of the following kinds:

Four unspotted dogs; of these one only (*i.e.* 1 in a total of 16) should produce uric acid.

Twelve more or less spotted dogs; of these four are pure bred and eight are hybrid. Of the four pure bred, one only (*i.e.* 1 in the total 16) should produce uric acid. Of the hybrid spotted, two (*i.e.* 2 in the total 16) should produce uric acid.

Only a certain number, of course, of the above animals of different composition would appear in a small litter and the proportions, moreover, would not necessarily be in close agreement with expectation.

It is suggested that the bitches *A*, *B* and *C* belong to the unspotted class; the bitch *D*, which closely resembles a pure Dalmatian, to the pure spotted class which does not produce uric acid; and, finally, the bitch *E* to the hybrid spotted class which does produce uric acid.

The accompanying scheme (p. 566) sets forth the inheritance of the two characters in the first and second generations. The criticism may be made that the animals in the scheme are represented as more heavily spotted than is justified by the photographs. Observation, however, demonstrates the fact that the spots, though slight in the young animal, increase in size and intensity, month by month, until the animal is 18 months or even two years old. The scheme is therefore designed to show, as correctly as possible, the markings of the mature animals.

In regard to the question as to whether the purine metabolism might not alter further with age, it may be pointed out that, at the completion of their experiments, the dogs of the second generation were approximately the same age as the dogs of the first generation at the beginning of their experiments. The metabolism of the latter did not change during the subsequent three months of experimental work.

Further matings and experiments no doubt would yield interesting results, but the main object of the research as formulated by the late author, namely, the mode of inheritance of the uric acid-producing character through two generations, has been determined.

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LXXII. THE EFFECTS OF VITAMIN-DEFICIENT DIETS ON THE ADRENALINE EQUILIBRIUM IN THE BODY.

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(Received July 4th, 1923.)

(A) INTRODUCTION.

IN 1919 McCarrison [1919] found that certain vitamin-deficient and otherwise unbalanced diets produced a marked increase in the size of the adrenal gland, which increase was associated with a larger store of adrenaline in the case of vitamin *B* deficiency. In fact he was at first inclined to believe that the appearance of oedema in the wet form of beriberi was to a large extent due to increased intracapillary pressure following on an increased output of adrenaline. In 1914 Funk and v. Schönborn [1914] found an increase in the blood sugar of pigeons fed on polished rice and in 1920 Funk [1920] suggested that it was due to increased adrenaline output. Anrep¹ observed in one or two cats which had been fed for a considerable time on vitamin *B*-deficient food that the blood-pressure was approximately 285 mm. Hg. while the animals were under anaesthesia. I have shown elsewhere² that deprivation of vitamin *B* from the diet of the adult rat leads almost at once to a pronounced intestinal stasis. These observations as well as the fact that adrenaline is a powerful gut inhibitor and that rats under advanced vitamin *B* deficiency show a number of signs resembling to some extent the effect of an increase in a sympathomimetic substance (*e.g.* nervousness, excitability, hair standing on end, etc.) made it seem very possible to me that the whole complex could be explained on a basis of hyperadrenalinemia [Gross, 1922]. Upon such a supposition, too, an explanation was afforded for the accumulation of large pigment-bearing cells confined to the subepithelial stroma of the colon which Keith [1914] described as occurring in advanced stasis in the human. For, granted that this pigment was taken in by phagocytes, or in some way obtained from the colon, (probably as a result of breaking down of aromatic decomposition

¹ Private communication.

² This will appear in a forthcoming publication.

products as suggested by Pick) these swollen pigment-bearing cells would find it impossible to pass lymphatics which were partially choked off by a muscularis mucosae kept in tonic contraction by increased adrenaline [Gunn and Underhill, 1914]. This explained, too, the lymphatic lakes and "soggy colon" of chronic intestinal stasis.

The experiments about to be described represent an attempt to confirm some of the statements made by these observers, and to determine directly the adrenaline content of the blood. 319 rats were used for the purpose.

(B) THE RELATION OF BODY WEIGHT TO ADRENAL GLAND WEIGHT AND ADRENALINE STORE.

The greater number of observations published on this subject are based on adrenal glands taken from animals which were either dead or dying. Furthermore, the diets largely used were grossly unbalanced so that the results in any case cannot be held to be those following pure vitamin deficiencies. The question seemed therefore worth reinvestigating in order to determine the changes in these glands which follow pure vitamin deficiencies on otherwise well balanced diets, and to make the adrenaline determinations on glands from adult rats which show evidences of vitamin deprivation, but are, on the whole, still in a relatively fair condition of health.

Table I.

Table of the diets used, showing the proportions of the ingredients in parts by weight. The butter was mixed with 2 % of its bulk of cod-liver oil. The heated casein was prepared by heating shallow layers of casein in a dry oven for 24 hours at a temperature of 110° C. The extracted casein was prepared by extracting casein with boiling 95 % alcohol for 4 hours. "Marmite" is a commercial preparation of autolysed yeast. The hardened fat used was hydrogenated cotton seed oil. The salt mixture was as follows in parts by weight:

	Sodium chloride	5.19		Calcium lactate	39.00		
	Magnesium sulphate	7.98		Ferrie citrate	3.54		
	Sodium acid phosphate	10.41		Calcium phosphate	16.20		
	Potassium phosphate	28.62					
Diet	Casein 20 parts	Starch 50 parts	Hardened fat, or butter, 10 parts	Salt mixture 5 parts	Marmite 5 parts	Lemon juice 5 parts	Water 30 parts
<i>P</i>	Casein	"	Butter	"	"	"	"
<i>A</i>	Heated	"	Hardened fat	"	"	"	"
<i>B</i>	Extracted	"	Butter	"	None	"	"
<i>C</i>	Casein	"	Butter	"	Marmite	None	"

The diets used in these experiments are shown in Table I. The average time during which the rats were kept on the various diets, as well as their average weights at the beginning and end of the experiment, are shown in Table II.

The rats were killed by a sharp blow on the head with a metal rod immediately followed by amputation of a fore limb. The blood was caught in a clean glass receptacle to be subsequently used for various experiments. This method of killing, besides being even more humane than coal-gas poisoning, gives an excellent yield of clean uncontaminated blood. The tissues are of course unaltered for histological purposes.

Table II.

The rats on *P* diet showed a steady increase in weight. The rats on *B* diet showed a consistent fall in weight. The rats on *A* and *C* diets presented a flattened growth curve which was more exaggerated in the case of *A* diet. The figures which give the average weights at the end of the experiment in this table include an average final fall in weight of 10 g. in the case of *A* diet, and an average final fall of 8 g. in the case of *C* diet.

	<i>P</i> diet	<i>A</i> diet	<i>B</i> diet	<i>C</i> diet
Average time on diet, in weeks	8	6	5	7
Average weight at beginning of experiment	184	205	228	193
Average weight at end of experiment	219	207	194	204

The method employed for estimating the adrenaline content of the adrenal glands was that of Folin, Cannon and Dennis [1912], which was largely used and found quite satisfactory by McCarrison [1921] and by Kellaway [1920]. The only modification which I adopted was to add the uric acid solution to my adrenaline extract in a quantity equal to that used in the standard tube. In this way the estimation of the small quantities of adrenaline found in a rat's adrenal glands was made possible. I found that the end-point in the colorimetric readings could be made considerably sharper by interposing an orange-coloured screen between the mirror of the colorimeter and the source of light. The latter should preferably be artificial. I used an Osram Daylight bulb, employing this light reflected from milk glass. The adrenal glands were obtained immediately after death and carefully dissected free from the surrounding fat. They were then weighed and the adrenaline estimated.

The results in this investigation are the averages from 350 glands obtained from 175 rats. A number of mice were also employed, but the experimental error was found too great to render these observations of any importance. Table III has been constructed entirely on the figures from rats' glands.

Table III.

	<i>P</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>B</i> deficiency followed by <i>P</i> diet
(a)	0.0253	0.0221	0.0188	0.0194	0.0174
(b)	0.0253	0.0228	0.0217	0.0208	0.0174
(c)	1.75	1.82	2.03	1.71	3.88
(d)	0.0442	0.0403	0.0382	0.0332	0.0675
(e)	0.0442	0.0415	0.0440	0.0356	0.0675

(a) represents g. of adrenal gland per 100 g. of highest body weight of rat.

(b) represents g. of adrenal gland per 100 g. of final body weight of rat.

(c) represents milligrams of adrenaline per g. of adrenal gland.

(d) represents milligrams of adrenaline per 100 g. of highest body weight of rat.

(e) represents milligrams of adrenaline per 100 g. of final body weight of rat.

Row (a) in Table III shows the comparison in weight between the glands of normal and vitamin-deficient rats. The numbers are all reduced to grams of gland per 100 g. of highest body weight of rat. The first fact to be noticed is that the adrenal glands of rats on the different vitamin-deficient diets decrease in size. This is most marked in vitamin *B* deficiency and least in vitamin *A* deficiency. Rondoni [1914, 1915], McCarrison [1919] and others [see McCarrison, 1921] have shown that vitamin and other dietary deficiencies cause a considerable increase in the size of the adrenal glands. McCarrison

conducted his experiments on monkeys, guinea-pigs and pigeons. My figures indicate the complete opposite for adult rats on pure vitamin deficiencies. The difference must depend either on the different species used, or the unbalanced diets used by these observers, or on changes during the last stages of vitamin deficiencies. Very likely the second reason given is the most important. I have noticed that the adrenal glands appeared noticeably enlarged and congested in several rats which I had deliberately kept on vitamin *B* deficiency to the point of death. The last stages of vitamin *B* deficiency are, however, undoubtedly complicated by the results of inanition. The average duration of my rats on vitamin *B*-deficient diet from which these figures are taken, is 5 weeks. One other point is worth noticing. In the normal rat, the adrenal gland weight is not in linear proportion to body weight. The heavier rats have as a rule slightly smaller adrenals per 100 g. of body weight than have the lighter or smaller rats. Attention must be paid to this point in comparing the effects of the various experimental diets. Table II shows that there is comparatively little difference between the average weights of my animals.

Row (b) represents grams of adrenal weight computed per 100 g. of final weight of rat. These figures show that the atrophy of the adrenal gland is not only absolute but relative as well.

Row (c) represents milligrams of adrenaline per g. of adrenal gland. It is seen that the adrenaline content of the adrenal glands of vitamin *B*-deficient rats shows a moderate relative increase in the store of adrenaline per weight of gland as compared to the content of adrenal glands from normal, *A*- and *C*-deficient animals. When, however, the gland content of adrenaline is reckoned per 100 g. of highest body weight of rat (row (d)), it is seen that an absolute diminution has taken place in vitamin *B*-deficient rats and even more so in *C*-deficient rats. Finally, when the adrenaline in the glands is computed against final body weight, which is nearest to the relation which it had during the life of the rat while on the experimental diet, it is seen that even these differences largely disappear (row (e)). *C* diet is the only deficiency which appears to produce any appreciable change. This change, however, is of the order of 19 %, and in view of the relatively high technical errors involved in determinations of this kind, too much significance must not be attached to it.

An entirely unexpected result was the relatively small glands found in rats which had been on a vitamin *B*-deficient diet for an average of 5 weeks and then placed on normal diet until the normal weight had been regained (usually about 2½ weeks). The average weight of gland per 100 g. of rat was 0.0174 g. The adrenal content was extremely high, 3.88 mg. per g. of gland. Worked out in term of mg. of adrenaline per 100 g. of highest weight of rat which was in this case equal to final weight, we arrive at the astonishing figure of 0.0675. This represents an absolute increase of 53 %.

Other points brought out were: (a) Unilateral adrenalectomised rats showed an increase in the store of adrenaline in the remaining gland. Thus,

whereas the normal gland showed 1.75 mg. the gland remaining after unilateral adrenalectomy contained 2.34 mg. of adrenaline per g. of gland. (b) Daily injections of adrenaline seemed to discharge, or perhaps decrease the formation of adrenaline in, the glands to a moderate extent, causing them to contain approximately 13 % less than on the same diet without the adrenaline injections. This was true for both normal and vitamin *B*-deficient diets.

Microscopic examination of the adrenal glands from normal rats as well as from those deficient in vitamins *A*, *B* and *C* respectively showed no appreciable change. The fixatives and stains used were 10 % neutralised formalin in saline, and osmic vapour [Cramer, 1918]. This investigation therefore shows that if the diet is otherwise well balanced, and if the adult rat is not kept on this diet until moribund, the changes in the adrenal glands both anatomically and functionally are comparatively slight.

(C) ATTEMPTS TO DETERMINE THE AMOUNT OF ADRENALINE PRESENT IN THE BLOOD AND SERUM OF NORMAL AND VITAMIN-DEFICIENT RATS.

This investigation was carried on at the same time, and on the same animals, as were used for the experiments described in the preceding section. It was not until the completion of the latter experiments that the statistical evidence derived therefrom pointed to the fact that when the store of adrenaline is compared to the weight of the animal, it is found that pure vitamin deficiencies produce on the whole very little, if any, disturbance in the adult rat. When the studies about to be described were being carried out, therefore, it was still on the assumption that vitamin deficiencies cause an upset in the adrenaline equilibrium with probably a resultant hyperadrenalinemia in the case of vitamin *B*.

The methods which I employed were as follows:

(a) Effect of serum on the isolated frog's pupil.

(b) Effect of blood and its derivatives on various other isolated organs such as rat's and rabbit's uterus and intestine, perfused rabbit's ear and perfused frog. In this study I co-operated with Professor A. J. Clark [unpublished].

It is, unfortunately, impossible to isolate adrenaline from the blood at present. Attempts at ultrafiltration and subsequent precipitation resulted in failure, I therefore turned my attention first to the use of the isolated frog's pupil. This method, which was first suggested by Meltzer and Auer [1904] and of which a critical survey was made by Schultz [1909], is not a specific test for the presence of adrenaline, for the mydriasis caused by this substance can also be brought about by other substances probably normally present in blood, *e.g.* pituitary extract. Nevertheless, in the hope that some idea of the relative amounts of mydriatic substance present in the serum would furnish useful evidence on the question, 63 rats were used for this purpose.

The blood was obtained in the manner already described. It was received

into a centrifuge tube and immediately spun for 5 minutes at 4000 revolutions per minute. The separated serum was pipetted off and 0.35 cc. run into each of several dwarf test-tubes. Frogs had meanwhile been pithed, the eyeballs carefully removed and placed upon a sheet of white paper with the pupils facing a constant source of light. It was planned to expose these pupils to the light for 5 minutes. The two diameters of the frog's ellipsoidal pupil were spanned with a pair of tool-maker's dividers and measured on a Vernier scale. The eyeballs were then immersed, one in each tube of serum. The same measurements were taken a half-hour later and one hour later, care being taken to carry out the whole process under similar conditions.

The areas of the pupils were computed from the measurements obtained, and the mydriatic effect of the serum expressed in terms of per cent. of the area increase over that found in the pupil just before immersion into serum. For controls, sera from normal rats were used.

A separate series of control experiments had brought out the following points:

(a) There was a well-marked variation in the reaction of the different frogs' pupils to the same serum, sometimes even the two eyes of the same frog gave a different reading for the same serum.

(b) On the whole the average of a fair number of eyes gave figures for known concentrations of adrenaline added to saline, which showed a distinct diminution in mydriatic power with increased dilution of the adrenaline. The utmost sensitivity, which I could obtain by this method, was a reaction to 1 in 8,000,000 adrenaline hydrochloride in saline.

Table IV.

Per cent. of area increase in frog's pupil: (a) after one half hour immersion in serum; (b) after one hour immersion.

	<i>P</i>	<i>A</i>	<i>B</i>	<i>C</i>	<i>B</i> deficiency followed by <i>P</i> diet
(a)	39	23	75	42	114
(b)	25	29	49	22	90

Table IV shows the results of these tests. It is seen that the serum of vitamin *A*-deficient rats has a somewhat lower mydriatic power, and that of vitamin *B*-deficient rats a considerably higher power, as compared to the controls. Also *B* rats which were subsequently fed on *P* diet for about two weeks showed an even greater mydriatic power of the serum. It will be remembered that the adrenaline content of these glands was considerably increased. This, in fact, is the only relation which I could find between the mydriatic power of the serum and the adrenaline store in the glands. Other points not shown in the table were the considerable decrease in mydriatic power of serum from unilateral adrenalectomised rats. The serum from rats which had daily injections of adrenaline showed neither in control nor in vitamin *B*-deficient rats, a departure from that found in the normal. In view

of the individual variations, the latter experiments as well as those on vitamin *A*-deficient animals, were rather too few in number to warrant more than a possible suggestion for future investigation.

It is seen, therefore, that if this increase in mydriatic power in the serum from vitamin *B*-deficient rats is due to adrenaline, the concentration in the serum should be well over 1 in 8,000,000.

To test this, Professor Clark and I compared the effects of fresh blood within 20 seconds from being shed, defibrinated blood, serum, plasma, ultrafiltrate of serum and plasma, and alcoholic and ether extracts of serum and plasma on the isolated organs previously mentioned in this section. Considering the fact that some of the organs, for example, rat's uterus, were occasionally sensitive to 1 in 500,000,000 adrenaline, well-marked reactions should have been obtained. It is true that shed blood very quickly develops vaso-constrictor and smooth muscle stimulating substances, but we endeavoured to obtain at least a quantitative difference between the blood of normal and vitamin *B*-deficient animals which would indicate a higher content of adrenaline in the blood of one of these groups.

Our results, which are described in greater detail in a separate publication, merely demonstrated the enormous variability of these fluids and tissues. We were, in short, unable to establish any definite differences between the adrenaline content of the blood of the normal and vitamin-deficient animals. Nor were we able to find any other characteristic differences in the properties of the blood of these animals in their effects on the isolated organs tested. It seems, therefore, highly improbable that the increased mydriatic effect of the serum which I found in vitamin *B*-deficient rats was due to a higher concentration of adrenaline.

(D) INVESTIGATIONS ON THE CARBOHYDRATE METABOLISM OF VITAMIN *B*-DEFICIENT RATS.

The chief interest which I had in the question was in so far as it would furnish indirect evidence on the relative amount of adrenaline in the circulation. Mr P. Eggleton and I are, however, making a detailed study of the carbohydrate metabolism in *B*-deficient rats which we hope will be published shortly. The present preliminary notice is based on the results from 48 rats. On the whole these are negative.

In 1914 Funk [1914] suggested that the anti-beriberi vitamin plays some part in carbohydrate metabolism. He based this view upon the fact that addition of carbohydrate to a standard beriberi diet caused the symptoms to appear earlier in pigeons. In the same year Funk and v. Schönborn [1914] found supporting evidence for this hypothesis in the discovery of a hyperglycemia and low liver glycogen in beriberi pigeons. They also observed that the administration of yeast tended to lower the blood sugar and increase the store of glycogen in the liver. In criticising these results, Vedder [1918] pointed out that overfeeding was probably the determining factor. In 1919

McCarrison [1919] stated that the absence of "anti-neuritic" food factors from the diet leads to an increase in the weight and adrenaline load of the adrenal gland and to a state of acidosis due to the imperfect metabolism of carbohydrates and the acid fermentation of starches. How far the increased weight of the adrenal gland and increased adrenaline store is due to deprivation of vitamin *B*, and how much to inanition, has been discussed in section (B). On the basis of McCarrison's results, Funk [1920] pointed out that increased adrenaline in the circulation was probably responsible for the hyperglycemia which he had obtained in another series of beriberi pigeons (bled out under A.C.E. mixture). It is noteworthy that Funk's figures show in this experiment a higher glycogen content in the beriberi livers than is seen in his normal controls. This is, of course, the opposite to what he and v. Schönborn had previously found. Very recently Mattill [1923] studied the respiratory quotient of vitamin *B*-deficient rats and concluded that there was no interference with the process of glucose combustion.

This condensed review presents the chief points brought forward which bear on the relation between vitamin *B* and carbohydrate metabolism. It was obviously important to reinvestigate this question in order to determine:

(a) Is pure vitamin *B* deficiency in the rat associated with an alteration in the carbohydrate metabolism?

(b) If so, what part in the cycle is affected, and in what manner?

The method which we employed was as follows: a large number of rats were fed on vitamin *B*-deficient diet and at different stages of the deficiency some were used for blood sugar and liver glycogen determinations. Experiments were also made on the blood sugar following the digestion of starch and that following the absorption of glucose. Since it is very difficult to obtain more than one sample of blood sugar from a rat without having recourse to anaesthesia, we killed the rat in each instance and relied upon statistical evidence of numbers for our conclusions. This eliminated most of the artefact which would otherwise be introduced into the actual sugar values. Also, much of the individual variation was thereby brought to a more reliable average. For blood sugar determinations we employed the MacLean [1919] 0.2 cc. method which we found quite reliable. For glycogen determinations we used Pflüger's method [1910], finally determining the hydrolysed sugar by Bertrand's method [1920].

The points investigated were as follows:

(a) *Blood sugar and liver glycogen from normal and vitamin B-deficient rats as they were taken out of the cage.* This is of course open to the objection that the blood sugar will depend upon when the animal had its last meal. These determinations were made, however, in order to compare the results with some work published by other authors who do not mention the precaution of preliminary starvation. The results obtained showed no appreciable difference for blood sugar, but a considerably lower liver glycogen in the *B*-deficient rats.

(b) *Blood sugar and liver glycogen from rats starved for 24 hours.* This is much more reliable since it does away practically entirely with carbohydrate absorption from the bowel. The liver glycogen in both normal and vitamin *B* rats was nil. The blood sugar was slightly higher in the *B*-rats than in the normal (0.09 g. % for normal, 0.117 g. % for *B*). It is possible, however, that additional numbers will reduce or remove this difference.

(c) *Rate of absorption of glucose from the alimentary tract of rats starved for 24 hours.* A glucose solution of known strength was introduced by catheter into the stomach, and blood sugar estimations were made at 15-minute intervals for one hour. No significant differences were found in the blood sugar curves from both groups of animals.

(d) *Introduction of a starch paste* gave us an indication of the rate of digestion as well as of absorption. Again no appreciable differences were found between normal and vitamin *B*-deficient rats.

In none of these experiments was a glycosuria found.

It is seen, therefore, that there is not as yet sufficient evidence to attribute to vitamin *B* deficiency an upset of carbohydrate metabolism, at any rate of the part studied by us thus far. Certainly the changes which we have found are insufficient to account for the marked general disturbances of the animal and do not afford adequate confirmatory support to the theory of hyperadrenalinemia. It is possible, however, that larger numbers of rats may reveal evidence which has not thus far been brought out.

(E) CONCLUSIONS.

The general conclusion which can be drawn from these investigations is that pure vitamin deficiencies set up very little alteration in the adrenaline equilibrium (*i.e.* relations between adrenaline store, gland weight and body weight) in the adult rat, providing the rat is not brought to the point of death on the diet. Such alterations as do occur can be explained on a basis of general tissue atrophy. The striking changes which have been recorded by other observers have probably been produced by the accompanying starvation and unbalanced dietary rather than by the deficiency in vitamins. Unless indeed, species so widely different as pigeons, guinea-pigs and monkeys are predisposed to alterations in adrenaline equilibrium to a much greater degree than is the adult rat. This seems very unlikely. Contrary to an upset in adrenaline equilibrium, the adrenaline store in the adrenal gland maintains an extraordinarily constant proportion to the weight of the rat. The suggestion forces itself upon one that this is no mere coincidence and that the remarkable way in which the store of adrenaline follows the body weight in its vicissitudes caused by the vitamin deficiencies argues very strongly for a finely controlled mechanism. It is then perhaps not surprising that I have been unable to obtain either direct or indirect supporting evidence of an unmistakable alteration in the adrenaline content of the blood.

I wish to tender my sincerest thanks to Sir Arthur Keith, to Professors E. H. Starling and J. C. Drummond, and to Dr Katherine H. Coward for their generous help and advice, as well as for the privilege of carrying out this work in their laboratories.

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LXXIII. THE OXYGEN CONTENT OF METHAEMOGLOBIN.

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SOME years ago one of us made measurements of the osmotic pressure of haemoglobin with a view to correlating changes in osmotic pressure of haemoglobin with haemolysis [Roaf, 1910, 1, and 1912]. In certain cases, notably in the action of alkali, there was a distinct relation between the effect of alkali on haemolysis and on the osmotic pressure of haemoglobin. In the case of acid, however, the results did not show so great an increase in osmotic pressure and it was suggested that some secondary change might have occurred.

The results were explained on the basis that haemoglobin acted as an amphoteric electrolyte with the formation of ionising salts, thus producing a corresponding rise of osmotic pressure and the establishment of an electrical potential.

Recently Mrs Wilson [1923] has published the results of experiments with carbon dioxide and acetic acid which confirm the result that acid causes a rise of osmotic pressure and she obtained higher pressures per 1 % of haemoglobin than were obtained by Roaf. Unfortunately, her paper does not indicate that spectroscopic examination, accompanied by reduction, was made when the pressure was high. In view of the fact that haematin may be recombined with protein [Menzies, 1894, 2], it is possible that the highest pressures obtained were due to some splitting of the haemoglobin and the subsequent fall due to recombination. It was for fear of this condition that dialysis experiments were not carried out with haemoglobin, but Mrs Wilson overlooks the fact that such experiments were carried out with serum proteins [Roaf, 1910, 2]¹.

¹ Mrs Wilson whilst confirming my results and agreeing with my conclusions states that they were based on slight evidence. The evidence was that haemoglobin although showing no evidence of decomposition into haematin gave higher osmotic pressures than could be accounted for by single molecules of haemoglobin. This evidence is good enough for the guarded statement: "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance sodium and 'haemoglobyl' ions."

The view that proteins form ionising salts is based on a much wider basis than one set of experiments. The starting point for this belief is the work of Emil Fischer in showing that

In the previous experiments methaemoglobin was tentatively considered to be the stable form of oxyhaemoglobin on the acid side of the isoelectric point although that view did not harmonise with the formation of alkaline methaemoglobin.

Buckmaster [1914] has published results in which he found that methaemoglobin formed by the action of hydroxylamine yields with hydrazine hydrate only half the nitrogen that is furnished by oxyhaemoglobin. The bearing of this point on the osmotic pressure measurements is that acid oxyhaemoglobin may have a high osmotic pressure but it readily passes into methaemoglobin with a lower osmotic pressure. If one could measure the osmotic pressure of oxyhaemoglobin in acid before methaemoglobin was formed, sufficient rise of pressure might be recorded to account for haemolysis by acid.

We thought this subject of sufficient interest to merit investigation by a different method. We measured the amount of oxygen given off when methaemoglobin was formed by the action of acetic acid on oxyhaemoglobin and compared this with the amount given off when another portion of the same oxyhaemoglobin solution was acted on by potassium ferrieyanide.

EXPERIMENTAL METHODS.

The method finally adopted was to use the principle of van Slyke's blood gas apparatus. In order to carry out a series of observations we used Haldane sampling bulbs for the containers. A tube over 76 cm. long was used to produce the vacuum (Fig. 1). On the upper end of this tube was a bulb (*B*) to receive the solution on evacuation. The lower end of the tube (*A*) was furnished with pressure tubing and mercury bulb in the usual manner. The Haldane sampling tube (*C*) was united with the bulb (*B*) by pressure tubing. After testing the apparatus to see that the taps did not leak the whole was filled with mercury. The haemoglobin solution was run in from a pipette through the upper opening of the sampling tube. The pipette was filled above the mark and the excess allowed to escape through the side outlet of the Haldane tube, so that all the connections up to the tap were filled with the solution. The tap was then turned through 180° so that the haemoglobin solution ran into the sampling tube above the mercury.

The connections were washed out with water through the side tube and the reagent added in the same way that the haemoglobin solution was added.

proteins are composed of amino acids. Other evidence was briefly quoted in the paper to which Mrs Wilson refers. She evidently overlooks the large amount of work published in this Journal from the Laboratory of the late Professor Benjamin Moore, F.R.S., which shows that the inorganic constituents are an integral part of the protein when they increase the osmotic pressure of the protein, otherwise the inorganic constituents would escape through the membrane. This work has been corroborated by other observers.

One point in connection with that work is frequently overlooked, viz. the importance of changes in the osmotic pressure of colloids on cell mechanics. If other cell proteins are as easily affected as haemoglobin considerable effects may be produced in cells by changes in osmotic pressure.

When ferricyanide was used the apparatus was immediately evacuated. After evacuation was completed the solution was collected in the bulb (*B*), the lower tap of the sampling tube was turned and the solution run out through the lower side tube for spectroscopic examination. The lower tap was then reversed and the mercury run into the Haldane tube.

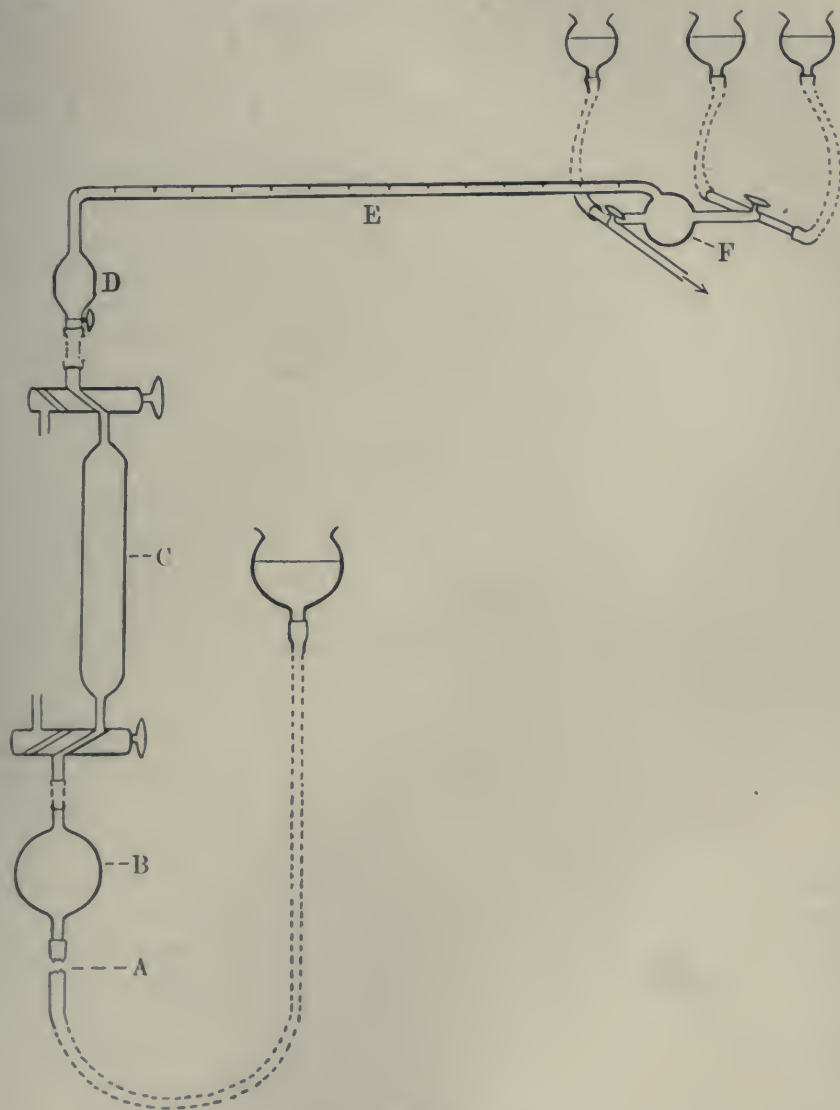


Fig. 1.

When acid was used, the reagent was added to the haemoglobin, both taps were closed, the tube disconnected, and left (usually for twenty-four hours at room temperature) until the acid had converted the oxyhaemoglobin into methaemoglobin. It was then re-connected and evacuated as described above.

The gas was analysed by running it into a tube containing alkaline water. This tube was connected to the Haldane tube by pressure tubing and the connections filled with water by running the alkaline solution through the upper side outlet of the Haldane tube. The upper tap was then reversed and by raising the mercury the gas was forced into the bulb (*D*).

By disconnecting the analysing tube from the Haldane tube and attaching a rubber tube with reservoir to the end which had been connected to the Haldane tube the gas can be forced along the measuring tube (*E*).

The absorption of gas was carried out in the far bulb (*F*). This bulb is furnished with two side tubes each with a right-angled tap so that either of two supply tubes, making four in all, could be connected with the bulb. The first reservoir contained the alkaline water for manipulation of the gas along the measuring tube. The second contained saturated sodium hydroxide for absorption of any carbon dioxide which escaped absorption by the alkaline water. The third contained concentrated pyrogallol which when run into the strong alkali in the bulb absorbed the oxygen. The fourth opening was attached to a waste tube for the purpose of washing out the apparatus.

The amount of oxygen given off was measured by the difference in volume after the action of strong alkali followed by alkaline pyrogallol, the usual assumption made in gas absorption analysis.

The haemoglobin solution was prepared as follows. The blood was centrifuged and the corpuscles twice washed with saline. The corpuscles were haemolysed by an equal volume of distilled water. At first as the result of osmotic pressure experiments we did not dare to use a preservative but later we found that toluene did not interfere with the results but actually improved them.

Caprylic alcohol was added to the alkaline water in the measuring tube to decrease the difficulty due to frothing.

RESULTS.

The results show unmistakably that addition of acid sufficient to convert oxyhaemoglobin into methaemoglobin liberates approximately half the amount of oxygen that would be given off by the same quantity of haemoglobin when acted on by ferrieyanide. A few of the results are given in tabular form.

DISCUSSION.

Before deducing that methaemoglobin contains only half the replaceable oxygen that oxyhaemoglobin contains we must examine all other possible explanations. As oxygen is given off the product formed by acid must contain less oxygen than oxyhaemoglobin and there is no source of oxygen other than the oxyhaemoglobin.

*Tabular Statement of Results in those experiments in which
toluene was used as a preservative.*

Date	Amount of haemoglobin solution cc.	Treatment of solutions	Amount of oxygen given off cc.		Percentage of oxygen capacity	Spectrum examination
Exp. 1	10	5 cc. saturated ferricyanide	1.68	av. of 2 readings	100	Methaemoglobin
19. ii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.54	" 2 "	45.8	Methaemoglobin + a doubtful trace of oxyhaemoglobin
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with air	0.22		6.5	Methaemoglobin + a trace of acid haematin
Exp. 2	10	5 cc. saturated ferricyanide	1.30	av. of 2 readings	100	Methaemoglobin
26. ii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.275	" 2 "	49	"
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with 90.1 % oxygen	0.57	accounted for by solubility of oxygen	22	"
Exp. 3	10	5 cc. saturated ferricyanide	1.37	av. of 5 readings	100	Methaemoglobin
5. iii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	1.355	" 2 "	49.5	"
	20	1.6 cc. 3 <i>N</i> acetic acid after equilibration with 56 % oxygen	0.42	accounted for by solubility of oxygen	15.3	"
Exp. 4	10	5 cc. saturated ferricyanide	1.53	av. of 3 readings	100	Methaemoglobin
12. iii. 23	20	1.6 cc. 3 <i>N</i> acetic acid	0.44		14.4	Methaemoglobin + acid haematin
A further set was started next day containing less acid with the following result:						
	20	1.4 cc. 3 <i>N</i> acetic acid	1.39	av. of 2 readings	45.4	Methaemoglobin
Exp. 5	10	5 cc. saturated ferricyanide	1.15	av. of 2 readings	100	Methaemoglobin
28. v. 23	20	1.4 cc. 3 <i>N</i> acetic acid	1.22		53	"

The average of these five experiments is 48.5 % of the oxygen capacity is given off when oxyhaemoglobin is converted into methaemoglobin.

We must first consider whether methaemoglobin is formed by the action of acid. There may be several forms of methaemoglobin, but at present it is recognised by its colour and absorption band. We have always examined the solution from the experiments in the following manner. We examined it spectroscopically for a band in the red near the *C* line. The solution was rendered alkaline and the change to alkaline methaemoglobin observed. The solution was then reduced and examined for reduced haemoglobin and reduced haematin. Any experiment the solution from which showed oxyhaemoglobin on first examination or reduced haematin on reduction was discarded.

We observed that the spectrum of methaemoglobin formed by the addition of acid lacked the two lines between *D* and *E* which are usually seen with methaemoglobin formed by ferricyanide. We think, however, that this does not indicate any real difference [compare Menzies, 1894, 1] because the addition of alkali to methaemoglobin formed by acid causes the two bands to appear. In our opinion the methaemoglobin formed by ferricyanide is a mixture of methaemoglobin and alkaline methaemoglobin: the two bands disappear with much less acid than is necessary to form methaemoglobin from oxyhaemoglobin.

The next point is whether the oxygen given off might have come from the incomplete formation of methaemoglobin. Any oxyhaemoglobin left unchanged might give off oxygen on evacuation. This alternative is shown not to occur because in those experiments in which oxyhaemoglobin was recognised spectroscopically the volume of oxygen given off was less than when no oxyhaemoglobin was found.

The alternative difficulty, namely, that acid haematin may be formed with the liberation of oxygen is negated by the result that if reduced haematin is seen after reduction the yield of oxygen is lower (see Exp. 4). In fact we made a few experiments to see if acid haematin contained less oxygen than oxyhaemoglobin. We found that if acid haematin is formed by using larger quantities of acid or by acid plus pepsin no oxygen is given off, thus indicating that acid haematin contains as much oxygen as oxyhaemoglobin.

In some experiments in which we treated oxyhaemoglobin with acid over mercury in a closed tube we did not find any gas above the solution. We wondered if this result were due to the gas remaining in a supersaturated condition or if there were two forms of methaemoglobin, one containing more oxygen than the other [Roaf and Smart, 1922]. Experiments were carried out to test this possibility. We prepared methaemoglobin by the action of acid during exposure to various pressures of oxygen. These solutions were run into our evacuating apparatus, and in all cases the amount of oxygen obtained was well below that liberated when oxyhaemoglobin was converted into methaemoglobin in a closed vessel and then evacuated. With high concentrations of oxygen more oxygen was observed (see Exps. 2 and 3), but the amount obtained corresponded to the amount that one would expect to find dissolved at the oxygen pressure used. In one case in which air was used above the solution the oxygen content of the air was increased by the amount that would correspond to the giving off of half the oxygen of the original oxyhaemoglobin. Therefore there is no evidence that there is a form of methaemoglobin containing more oxygen at higher oxygen pressures.

Having discussed the alternative explanations of our results we wish to draw attention to some of the difficulties of the experiments. As suggested above, too much or too little acid will give results too low because acid haematin fails to give off oxygen and oxyhaemoglobin was associated with a lower yield. Therefore only those experiments in which the right amount of acid was used gave results worth recording. Incomplete saturation of the oxyhaemoglobin will give as a result less than half of the oxygen content observed by the ferrieyanide method. Bacterial action will cause a loss of oxygen, and before the use of toluene as a preservative lower results were obtained. Chemical action, apart from bacterial action, might cause some loss of oxygen, so that is another possible cause of low results. In the evacuation of the methaemoglobin solution the solution to which acid had been added was viscous and it frothed badly. This frothing was one of our difficulties, and it was less easy to get off all the oxygen than from the methaemoglobin formed by ferrieyanide. We attempted to use horse corpuscles obtained through the courtesy of Dr O'Brien of Messrs Burroughs, Wellcome and Company (to whom our thanks are due), but we abandoned them for bullocks' corpuscles, because the horse haemoglobin became practically solid after the action of acid. We are not certain whether there is a difference due to the species or whether it is due to the absence of calcium in the case of the horse corpuscles, which were obtained from oxalated blood and washed with oxalated saline.

In view of these various reasons why the amount of oxygen obtained might be less than the theoretical amount we consider that our results show a very good approximation to 50 % of the dissociable oxygen of oxyhaemoglobin.

Hüfner and Külz [1883] are frequently quoted as having proved that methaemoglobin contains the same amount of oxygen as oxyhaemoglobin. Their experimental method was to treat solutions of oxyhaemoglobin and methaemoglobin with urea and nitric oxide. Their results show that the same amount of nitrogen is given off in each case, but that may be due to only part of the oxygen of oxyhaemoglobin being concerned in the reaction. Küster [1910], in discussing this problem, points out that hydrocyanic acid will combine directly with methaemoglobin but not with haemoglobin. Oxyhaemoglobin reacts slowly with hydrocyanic acid. He concludes that methaemoglobin is intermediate between oxyhaemoglobin and haemoglobin.

Methaemoglobin is formed by such a variety of agents, some oxidising and some reducing [for instance palladium-hydrogen, Hoppe-Seyler, 1877, 1878], that one is led to the belief that it is the stable form in acid solution. Alkaline methaemoglobin also seems to be a stable form on the alkaline side, but it is easily converted into haemoglobin by reducing agents without passing through the form of oxyhaemoglobin (Haldane).

If the deduction made in this paper is correct the equations given by Haldane [1898] for the action of ferricyanide on haemoglobin and on oxyhaemoglobin should be rewritten with two molecules of ferricyanide instead of four. If there were a reasonable method for the estimation of ferrocyanide in the mixture this might be put to the test of experimental verification.

$\text{HbO}_2 + 2\text{K}_3\text{Fe}(\text{CN})_6 + 2\text{NaHCO}_3 = \text{HbO} + 2\text{K}_3\text{NaFe}(\text{CN})_6 + 2\text{CO}_2 + \text{H}_2\text{O} + \text{O}_2$,
and

$\text{Hb} + 2\text{K}_3\text{Fe}(\text{CN})_6 + 2\text{NaHCO}_3 = \text{HbO} + 2\text{K}_3\text{NaFe}(\text{CN})_6 + 2\text{CO}_2 + \text{H}_2\text{O}$.

CONCLUSIONS.

Oxyhaemoglobin on conversion into methaemoglobin loses half of its dissociable oxygen. This is shown in our experiments by the actual liberation of oxygen as the result of the action of acid. In Buckmaster's experiments the evidence is that hydrazine hydrate liberates only half as much nitrogen when it reduces methaemoglobin as when it reduces oxyhaemoglobin.

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LXXIV. ON GLUTATHIONE. IV. CONSTITUTION.

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and Caius College*).

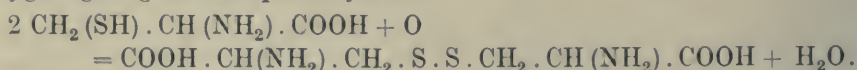
From the Biochemical Laboratory, Cambridge.

(Received July 12th, 1923.)

GLUTATHIONE was isolated from yeast and from various animal tissues by Hopkins [1921]. It is a white amorphous powder, non-hygroscopic when pure. It is very soluble in water, but insoluble in the usual organic solvents. Heated in a capillary tube, it softens at 165–167°, and melts with evolution of carbon dioxide at 182–185°. The yield of glutathione from fresh tissues was about 0.01–0.02 %; from yeast, the usual source, a similar amount was obtained.

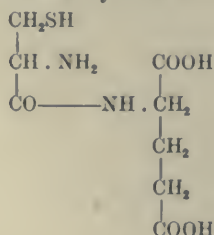
On the following evidence, Hopkins [1921] considered glutathione to be a dipeptide of glutaminic acid and cysteine. The elementary analysis agreed with the formula $C_8H_{14}O_5N_2S$, which such a dipeptide would possess. The peptide structure was shown by the fact that the primary-amino nitrogen, as determined by the method of Van Slyke [1911], was half the total nitrogen, and was, moreover, doubled by hydrolysis. Finally, acid hydrolysis of glutathione gave *d*-glutaminic acid and *l*-cystine.

The sulphydryl group of cysteine is readily oxidised, even by atmospheric oxygen, giving the disulphide cystine.

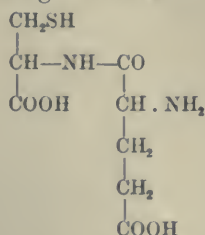


Similarly, glutathione can be obtained in two forms: one—"reduced glutathione"—containing cysteine: the other—"oxidised glutathione"—containing cystine. The importance of glutathione in tissue oxidation processes depends on the ready inter-convertibility of these two forms.

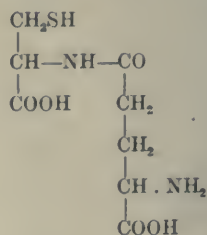
A dipeptide of glutaminic acid and cysteine must possess a structure represented by one of the following formulae:



I



II

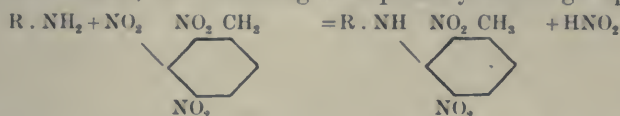


III

Our problem was therefore to find which of these three formulae is that of glutathione.

The first stage was to find through which amino group—that of glutaminic acid, or that of cysteine—the linkage of the two amino-acids was brought about. For this purpose two methods were adopted, both of which had previously been used by Barger and Tutin [1918] in determining the constitution of carnosine.

In the first method, use was made of the power possessed by 2.3.4- and 2.4.5-trinitrotoluene, of condensing with primary amino groups.

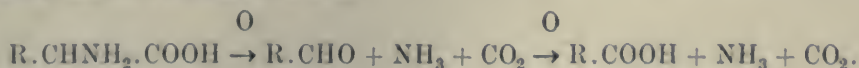


The condensation product of glutathione and 2.3.4-trinitrotoluene, on hydrolysis, yielded free cystine. It must be concluded, therefore, that the trinitrotoluene had reacted with the amino group of the glutaminic acid part of glutathione, and that the amino group of cysteine had formed part of the peptide linkage. From the hydrolysis mixture, a yellow substance was also isolated: this was probably the condensation product of glutaminic acid with trinitrotoluene. Its identity was not definitely ascertained however, owing to the failure of all attempts to obtain a satisfactory condensation between trinitrotoluene and free glutaminic acid.

The second method consisted in the replacement of the free primary amino group of the dipeptide by hydroxyl, and identification of the hydroxy-acid obtained after hydrolysis of the resulting compound. In this way, α -hydroxyglutaric acid was isolated, a result confirmatory of that obtained by the first method.

These experiments agree, then, in showing that in glutathione, the free primary amino group is in the glutaminic part of the molecule, and that it is the amino group of the cysteine which is involved in the peptide linkage. Formula I, therefore, is not that of glutathione, but the evidence so far does not differentiate between Formulae II and III.

It remained, therefore, to determine which of the two carboxyl groups of glutaminic acid was, in glutathione, condensed with the amino group of cysteine. For this purpose, use was made of an investigation by Dakin [1905], who found that α -amino-acids, oxidised by means of hydrogen peroxide in presence of a trace of iron salt, gave carboxylic acids containing one carbon atom less than the original amino-acid. The course of the reaction, he has shown, may be represented thus:



Glutaminic acid, oxidised in this way, yielded succinic acid [Dakin, 1908]. Formula II, is that of a γ -amino-acid, Formula III, of an α -amino-acid. A compound of Formula III, oxidised by hydrogen peroxide, would be converted to a substance containing the succinyl radicle in place of the original glutaminyl radicle. This compound would probably be, not succinyl-cysteine,

but succinyl-cysteic acid, the sulphydryl of the cysteine having been oxidised to the sulphonic acid group. After, and only after, hydrolysis of this compound, would free succinic acid be obtained as one of the products.

A compound of Formula II, on the other hand, must behave rather differently. Even if the $>\text{CH.NH}_2$ group be oxidised, the resulting carbonyl must, by continued oxidation, lead to a breakage of the chain, and the immediate formation of succinic acid *before* hydrolysis.

Experiments were carried out on these lines, and it was found that simple oxidation of glutathione by hydrogen peroxide produced no trace of succinic acid, whereas after hydrolysis of the oxidation product, succinic acid was isolated in a yield amounting to 50 % of the theoretical. It is therefore concluded that the structure of glutathione is represented by Formula III.

The ultimate proof of constitution lies in the synthesis, by known reactions, of the substance under consideration. Hence, until glutathione has been synthesised—a task on which the authors are at present engaged—the conclusions set forth in this paper must be regarded as being in no way final. The analytical evidence, however, which is briefly summarised below, appears to be quite strong enough to justify the deductions drawn from it.

1. Glutathione condenses with 2.3.4-trinitrotoluene, its free amino group being thereby bound. Hydrolysis of the resulting compound gives cystine, but no free glutaminic acid.

2. The free amino group of glutathione having been replaced by hydroxyl, a compound is obtained which, by hydrolysis, gives α -hydroxyglutaric acid.

These entirely different methods agree in showing that in glutathione, it is the amino group of the cysteine which is concerned in the peptide linkage.

3. Oxidation of glutathione by hydrogen peroxide yields succinic acid only after hydrolysis of the oxidation product.

Hence glutathione is itself an α -amino-acid, and of Formulae II and III, only the latter satisfies this condition. Moreover, this result affords additional confirmation of the deductions drawn from the previous experiments, since a compound of Formula I could not yield succinic acid under the circumstances.

Owing to the low concentration in which glutathione is found, and the long and complicated method necessary for its separation, the amount available for this work was very small. The whole of the experiments described were carried out on rather less than 2 g. of material.

EXPERIMENTAL.

Condensation of Glutathione with 2.3.4-Trinitrotoluene.

0.3 g. of "oxidised" glutathione and 1.0 g. of 2.3.4-trinitrotoluene, dissolved in 40 cc. of 60 % ethyl alcohol, were heated under a reflux condenser, on the water-bath, for two hours. After ten minutes the liquid began to turn yellow, and the colour gradually deepened. The clear solution was evaporated to dryness on the water-bath: absolute alcohol was added and evaporated off to

remove the last traces of water. The residue was extracted three times with dry benzene, to remove unchanged trinitrotoluene. The yellow crystalline powder remaining behind was dissolved in hot water. The solution was filtered and the filtrate was evaporated to dryness on the water-bath. The yellow residue was washed with absolute alcohol, in which it was only very slightly soluble. Absence of glutathione from this residue was shown by its inability to give any precipitate with mercuric sulphate in acid solutions.

The condensation product thus obtained was a yellow semi-crystalline substance, very soluble in water, only slightly soluble in ethyl alcohol, insoluble in ether and benzene. Heated in a capillary tube, it softened at 115° and melted sharply with decomposition at 202° (uncorr.). Yield 75 % of the theoretical.

Analysis:

0.1113 g. dried at 100°; 0.1695 g. CO₂; 0.0501 g. H₂O.

Found C = 41.54 %; H = 4.98 %.

Calculated for (C₁₅H₁₇O₉N₄S)₂ C = 41.94 %; H = 3.97 %.

Nitrogen was estimated by a modified micro Kjeldahl [Stewart, 1923].

10.0 mg. contained 1.314 mg. Nitrogen N = 13.14 %.

Calculated for (C₁₅H₁₇O₉N₄S)₂ N = 13.06 %.

Hydrolysis of the Condensation Product.

0.4 g. of the condensation product were dissolved in 15 cc. of 30 % sulphuric acid and boiled under a reflux condenser for four hours. At the end of this time, the liquid was cooled, and thoroughly extracted with ether. The deep yellow ethereal extracts, on evaporation left a yellow residue, readily soluble in alcohol and in water, which was probably the condensation product of glutaminic acid and trinitrotoluene. The almost colourless aqueous layer was diluted to 60 cc. with distilled water, and excess of mercuric sulphate solution was added. The voluminous white precipitate was separated by centrifuging, and washed repeatedly with distilled water. It was then suspended in water, and decomposed by hydrogen sulphide. The filtrate from mercuric sulphide was boiled until all the hydrogen sulphide had been expelled. The colourless solution then gave the following reactions:

1. A violet coloration (when neutralised) with triketohydrindene hydrate.
2. A vivid permanganate coloration with sodium nitroprusside in presence of ammonia.
3. A black precipitate of lead sulphide on prolonged boiling with lead acetate and sodium hydroxide.

It was therefore concluded that the solution contained cysteine.

A slight excess of baryta water was added, and after removal of the barium sulphate, the solution was aerated until the nitroprusside reaction was negative—i.e. until the conversion of cysteine to cystine was complete. Barium was then quantitatively removed by addition of sulphuric acid, barium

sulphate was filtered off, and the clear liquid concentrated on the water-bath. On standing, cystine separated in typical hexagon-shaped plates, and on further concentration of the mother liquors, in acicular aggregates.

Nitrogen by micro Kjeldahl:

8.7 mg. contained	·999 mg. Nitrogen	N = 11.48 %.
Calculated for	$C_6H_{12}O_4N_2S_2$	N = 11.66 %.

De-amination of Glutathione.

0.5 g. of glutathione was dissolved in 10 cc. of water, and 2.0 g. of freshly prepared silver nitrite were suspended in the solution. The liquid was cooled in ice, and 20 cc. *N* HCl were added gradually with constant stirring. The mixture was left in ice for one hour, and was then allowed to stand overnight at room temperature. The precipitated silver chloride was filtered off, and the filtrate was found to be free from silver. The liquid was extracted with ether, and the dried ethereal extracts were evaporated to dryness on the water-bath. There was no residue.

Hydrolysis of De-aminated Glutathione: Isolation of α -Hydroxyglutaric Acid.

An equal volume of 35 % hydrochloric acid was added to the solution containing the de-aminated glutathione, and the mixture was boiled under a reflux condenser for four hours. After cooling, the hydrolysis mixture was thoroughly extracted with ether, and the combined ethereal extracts, dried over anhydrous sodium sulphate, were evaporated on the water-bath. There remained an oily liquid, which, on cooling, solidified to a deliquescent, crystalline mass. Yield 0.10 g. It had a fruity odour: was strongly acid, was readily oxidised by potassium permanganate, and gave no coloration with triketohydrindene. Tests for sulphur and nitrogen were completely negative. The substance melted well below 100°, but owing to its deliquescent nature, no accurate determination of the melting point could be made.

For analysis, the calcium salt was prepared by addition of calcium chloride in slight excess to the neutralised solution of the acid in slightly dilute alcohol. The precipitated salt was separated by centrifuging, washed thoroughly with alcohol and finally with ether. It was dried at 37°. A weighed quantity of the salt was transferred to a centrifuge tube, and dissolved in dilute hydrochloric acid. A slight excess of saturated ammonium oxalate was added, and the liquid was made alkaline with ammonia. It was allowed to stand one hour, and was then centrifuged. Most of the supernatant liquor (all but about 0.3 cc.) was siphoned off, and the precipitate was washed three times with 2 % ammonia by the same process. The calcium oxalate was then dissolved in 2 cc. *N* H_2SO_4 , warmed on the water-bath to 60°, and titrated with 0.01 *N* potassium permanganate from a micro-burette.

6.2 mg. of calcium salt required 6.27 cc. of 0.01 N KMnO_4 ;

Ca = 20.22 %.

Calculated for calcium α -hydroxyglutarate, $\text{Ca} \cdot \text{C}_5\text{H}_6\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$;

Ca = 20.51 %.

Oxidation of Glutathione.

0.5 g. of "oxidised" glutathione was dissolved in a little water, and the solution was made slightly alkaline with ammonia. A trace of ferrous sulphate was added, and then 25 cc. of 5 % hydrogen peroxide. After half an hour at 70° , evolution of gas had ceased, and a further 25 cc. of 5 % hydrogen peroxide were added, the heating being continued to a total of one hour. The solution then gave no coloration with triketohydrindene, whereas glutathione itself gives a strongly positive reaction. Evidently the free amino group had been removed by the oxidation. Further, mercuric sulphate produced no precipitate. After cooling, the solution was acidified with hydrochloric acid, and thoroughly extracted with ether. The ethereal extracts, dried and evaporated, left no residue. No free succinic acid, therefore, had been formed by the oxidation.

Hydrolysis of the Oxidation Product.

The solution of the oxidation product was evaporated to dryness to remove all traces of ether and hydrogen peroxide. The residue was dissolved in 20 cc. of 30 % hydrochloric acid, and boiled under a reflux condenser for three hours. The solution was cooled and extracted with ether. The combined ethereal extracts were dried and evaporated on the water-bath, when there remained a white crystalline solid, in amount equal to 40 % of the theoretical yield of succinic acid. That this substance was succinic acid was shown in the following ways:

1. It formed bunches of thick prismatic needles, identical in form with those of succinic acid.
2. It was not readily soluble in water, and the aqueous solution was strongly acid.
3. It gave no coloration with triketohydrindene.
4. The ammonium salt, distilled with zinc dust gave the pyrrole reaction with a pine splint moistened with hydrochloric acid.
5. Heated with resorcinol and concentrated sulphuric acid, it gave the typical fluorescent solution.
6. Heated alone, it melted and sublimed in feathery needles, and produced a choking odour.
7. Its neutral solution gave a precipitate with ferric chloride solution.
8. From neutral solution, its silver salt was precipitated by the addition of silver nitrate.
9. The acid, after purification through the silver salt, melted at 180° (uncorr.) and the melting point was not altered by the admixture of pure succinic acid.

For analysis, a weighed amount of the acid, purified through the silver salt, was dissolved in water, and titrated with standard alkali from a micro-burette. From this neutral solution, the silver salt was precipitated, and after thorough washing was dissolved in nitric acid and titrated with standard potassium thiocyanate.

1. 15.0 mg. of the acid required 5.050 cc. 0.05N NaOH
[\equiv 14.89 mg. succinic acid].

The silver salt required 6.3 cc. 0.04N KCNS
[\equiv 14.87 mg. succinic acid].

2. 4.4 mg. of the acid required 1.495 cc. 0.05N NaOH
[\equiv 4.41 mg. succinic acid].

The silver salt required 1.850 cc. 0.04N KCNS
[\equiv 4.36 mg. succinic acid].

Our thanks are due to Prof. F. G. Hopkins, F.R.S., for the supply of material and for the kind interest he has taken in this work, also to Prof. G. Barger, F.R.S., for the specimen of 2, 3, 4-trinitrotoluene used in this work, and for helpful criticism. Two of us (J. H. Q. and H. E. T.) are indebted to the Department of Scientific and Industrial Research for grants which have enabled us to carry out this work.

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LXXV. ON THE PRESENCE OF MALTASE IN GERMINATED AND UNGERMINATED BARLEY.

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It has long been known that when starch is hydrolysed in presence of malt diastase under certain conditions, glucose is one of the products. Some chemists have attributed this production of glucose to the presence of the enzyme, maltase, in the malt. Thus Effront in 1899, dealing with the conversion of maltose into glucose by enzyme action, remarks [1899]: "L'infusion de malt agit très peu sur le maltose, mais le malt concassé agit énergiquement sur les sirops de maltose, qui se transforment en sirop de dextrose."

It is, however, on record that, when starch is hydrolysed in presence of enzyme preparations which do not contain maltase, glucose is, under certain conditions, produced. It was stated by Ling and Baker [1895] in 1895 that glucose is one of the hydrolytic products of the action on potato starch paste of the diastase prepared from kilned malt. In a subsequent paper [1897] these authors observed that they had now satisfied themselves that the production of glucose in the manner indicated was in no way connected with the kilning of the malt. However, Ling and Davis later [1902] showed that when an aqueous solution of precipitated diastase is heated at 65°, or at a few degrees above this temperature, and the solution allowed to act on starch paste, glucose is invariably present among the final products of hydrolysis. Here we have a distinct confirmation of the original observation of Ling and Baker.

Brown and Millar [1899] showed that the so-called stable dextrin can be partially hydrolysed in presence of malt diastase at 55° and that glucose is among the products. Ling [1903] showed that the malto-dextrin- α of Ling and Baker also yields about 10 % of glucose when hydrolysed in the presence of malt diastase at 55°.

Davis and Ling [1904], who measured the quantity of glucose formed from heated diastase solutions, showed that the maximum quantity of the sugar just mentioned is produced when the diastase solution is heated at 68–70°, a temperature at which maltase is inactive. Ling and Rendle [1904] showed that commercial concentrated malt extracts contain from 17–22 % of glucose. It will be seen therefore that glucose may be produced from starch and some of its hydrolytic products by diastase preparations not containing maltase.

We have indeed proved that no glucose is produced when precipitated malt diastase is used as the hydrolysing agent for starch unless the diastase has been heated to 65° in aqueous solution. Moreover, under all conditions, whether its aqueous solution has been heated or not, no glucose is produced by its action on maltose.

It appears to be otherwise with malt extract, Maquenne having recently shown [1923] that glucose is produced when maltose is treated with malt extract. As a result of our present experiments we find that maltase is present in the extract of both green and kilned malt and also in ungerminated barley. In the latter case, however, the enzyme is present in a form in which it cannot be extracted by water, for barley extract is without action on maltose whilst barley grist when added to maltose solution causes hydrolysis to glucose. Maltase is an endogenous enzyme which is very sensitive to reagents and very difficult to isolate. It is also thermo-labile, being destroyed below 70°.

The method adopted in the experiments we have carried out to demonstrate the presence of maltase was in the case of malt to act on a solution of maltose with an extract of the malt, or in the case of barley, where the enzyme cannot be extracted with water, with the barley grist. After a convenient period of incubation the percentage of maltose hydrolysed was determined by estimating gravimetrically the glucose formed as phenylosazone according to the method described by Davis and Ling [1904].

Dealing first with the malt experiments, five different kinds of malt were employed. No 1 was a green malt made from English two-rowed barley. No. 2 a green malt made from a foreign six-rowed barley, and Nos. 3, 4 and 5, were all kilned malts with diastatic powers, 23°, 52° and 102° (Lintner) respectively. The malt extract used was prepared by digesting one part of the finely ground malt with ten parts of water containing a little toluene at the ordinary temperature for 24 hours.

To 100 cc. of the filtered extracts thus obtained from the above-mentioned malts were added 2.12 g. of maltose and the solutions were incubated at 50°. A convenient volume of each of the malt extracts with a little toluene was also incubated side by side without the addition of maltose to serve as blanks. After a period of 45 hours the amount of glucose in each of the solutions was estimated gravimetrically as follows:

20 cc. of each of the solutions containing about 3 % carbohydrates were measured out into clean 25 cc. test-tubes and 1 cc. of phenylhydrazine and 1.5 cc. of 50 % acetic acid added to each. The solutions were then heated in a boiling water-bath for exactly one hour. At the end of this period the liquid was carefully decanted on to a tared Gooch crucible which was previously warmed with boiling water. Care was taken to decant the supernatant liquid on to the filter before transferring the glucosazone. The precipitate of glucosazone was then washed with 25 cc. of boiling water and then dried in a steam oven until the weight was constant. In the following results the difference in weight of the glucosazone between the blanks and actual experiments repre-

sents the amount of glucose due to the hydrolysis of maltose. The amount of glucose corresponding to the glucosazone was obtained by multiplying the weight of the glucosazone by the factor 1.98.

Table I.

Malts used	Wt. of the osazone from 20 cc.		Difference for 20 cc. g.	Wt. of maltose hydrolysed per 100 cc. g.	% of maltose hydrolysed
	With maltose g.	Without maltose g.			
(1) English green malt	0.1990	0.1808	0.0182	0.1712	8.07
(2) Foreign green malt	0.1970	0.1596	0.0374	0.3518	16.59
(3) Kilned malt (Californian). D.P. 23°	0.2135	0.2017	0.0118	0.1110	5.23
(4) Kilned malt (English). D.P. 52°	0.3016	0.2768	0.0248	0.2332	11.00
(5) Kilned malt (Danubian). D.P. 102°	0.2818	0.2675	0.0141	0.1310	6.18

These results show conclusively that a soluble maltase is present in malts made under different conditions. The power of the enzyme appears however not to vary directly with the diastatic power of the malt expressed on the Lintner scale, but according to the temperature and rate at which the moisture has been expelled on the kiln. To account for the presence of a soluble maltase three possibilities suggested themselves.

The enzyme may exist in the barley before germination; or it may be elaborated during the processes of germination; or it may exist in the barley in an insoluble form before germination by which it may be rendered soluble. Further experiments were carried out to settle these points and the results show that the last view is the most probable. In the barley experiments starch paste was used as the substrate instead of maltose as it is known that barley diastase does not give rise to any other sugar than maltose [cp. Baker, 1902]. Our own experience, moreover, indicates that no maltase is present in barley extract. Accordingly, barley grist was employed for converting the starch paste and under these circumstances a considerable amount of glucose was formed.

The experiments were carried out as follows. To 20 cc. of 2-3 % starch paste 3 g. of the finely ground barley grist were added and the mixture with the addition of a little toluene incubated at 50° for 24 hours. At the end of this period the liquid was filtered and the glucose estimated as before in 20 cc. portions of the filtrate. Blank experiments were also carried out without the addition of starch, but no glucosazone was obtained from these.

In the following table are given the results of five such experiments.

Table II.

Specific gravity of the conversion liquid	Wts. of the osazone from 20 cc. g.		Difference g.	Glucose per 100 cc. g.	% of maltose hydrolysed on the total solids
	With starch	Blank			
1008.65	0.015	Nil	0.015	0.1485	6.41
1010.30	0.053	..	0.053	0.5248	19.00
1008.10	0.020	..	0.020	0.1980	9.13
1007.50	0.012	..	0.012	0.1188	5.94
1009.90	0.022	..	0.022	0.2178	8.24

From these experiments we can safely infer that there is an insoluble maltase present in the raw barley, and that this undergoes modification during germination and is rendered partially soluble. The soluble maltase content of malt varies according as it is low dried or high dried. Green malts especially contain considerably more maltase, as the enzyme is probably partially destroyed during the kilning process.

CONCLUSIONS.

1. It is shown that malts, whether green or kilned, obtained by germinating barley contain an enzyme capable of hydrolysing maltose. The power of the enzyme depends, *caeteris paribus*, on the temperature and the way in which the malt has been heated on the kiln.

2. Diastase preparations obtained by precipitating a cold water extract of malt with alcohol do not contain maltase, as this enzyme is destroyed by the alcohol.

3. Ungerminated barley contains an enzyme capable of converting maltose into glucose. It cannot be extracted with water, but its activity is demonstrated by allowing ground barley (grist) to act on maltose.

Our thanks are due to W. J. Harper (Assistant in the Department) for carrying out some of the analyses connected with this work.

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LXXVI. THE INFLUENCE OF THE ANTENATAL FEEDING OF PARENT RATS UPON THE NUMBER, WEIGHT AND COMPOSITION OF THE YOUNG AT BIRTH.

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(Received July 13th, 1923.)

IN our previous experiments [1923, 2], as well as in the experiments of Korenchevsky [1921, 1922], we came to the conclusion that, as the result of a properly constituted diet taken by the mother during pregnancy or lactation, rickets and general disorders of nutrition in the offspring can be mitigated or even prevented for a considerable time; or *vice versa*, a diet, improperly constituted, may cause or aggravate serious nutritional diseases in her young. These facts were proved by experiments on young rats immediately after weaning and also when about 2-3 months old.

In the present investigation we studied the following questions:

1. Has the mother's diet before and during pregnancy any influence on the weight and number of the young, as well as on their chemical composition, at the time of birth?
2. Has the father's diet before conception any influence upon the same factors?
3. To what cause were due the striking differences found after weaning [Korenchevsky, 1921, 1922; Korenchevsky and Carr, 1923, 2] in the composition of the skeleton of the young born of mothers on normal diets. For instance, is the storage of calcium and phosphorus in the foetuses increased when the mother is on a diet rich in these elements and in cod-liver oil?

The experiments were conducted on 30 litters of rats. The technique of the experiments and the diets used were described in Korenchevsky's previous papers [1921, 1923]. The mother rats were fed on the following basal diets [for details see Korenchevsky, 1922, 3, p. 7]:

D 1 consisting of milk, cabbage, oats, bran and white bread.

N 2, containing a liberal amount of fat-soluble factor in the form of butter and cod-liver oil, 0.52 % Ca and 0.5 % P (in fresh diet).

— A, a diet similar to N 2, but deficient in fat-soluble factor, the only source of fat being cotton seed oil, and containing only 0.25 % Ca in the fresh diet.

In each series of experiments the mother rats were taken from the same litter and the father rats from another litter. After weaning, the young rats were put on special diets: some were put on *N* 2 diet and were kept on this the whole time. Rats are usually sterile on *-A* diet, therefore, before conception the mother rats in this group received *D* 1 diet and only from the day of conception or about one week before this, were put on *-A* diet on which they were kept during the whole pregnancy. The father rats in this group were also kept on *D* 1 diet and about a fortnight before conception were put on *-A* diet.

The experiments were so arranged that the mothers and fathers in each series were approximately of the same age at the day of conception. The pregnant rats were carefully watched during the last period of pregnancy and immediately after birth, or in some cases 1-2 hours later, the young were killed by breaking their necks. After the stomachs and intestines had been removed the young were weighed and used for estimation of water, calcium (McCrudden's method), phosphorus (Neumann's method) and nitrogen (Kjeldahl's method).

The results of our experiments are seen from Tables I and II. The different diets of father and mother rats are clear from Table II. The number, weight and water content of the young were estimated in all 30 litters, but only in 13 of these were the total calcium, phosphorus and nitrogen determined.

Table I. *Average Weights of Mothers.*

Diets of mothers		Weight of mothers	
Before conception	During pregnancy	Four weeks before birth	After birth
<i>N</i> 2	<i>N</i> 2	179	212
<i>D</i> 1	<i>-A</i> or <i>D</i> 1	149	171

Table II. *Average Number, Weight and Composition of the Young at Birth.*

No. of group	Diets				Average number of young in each litter†	Average weight*			H ₂ O %	In young					
	Of father	Of mother		Number of litters		Of the whole litter	Of one fresh young	Of one dry young		Ca %		P %		N %	
		Before conception	During pregnancy							Fresh	Dry	Fresh	Dry	Fresh	Dry
I	N 2	N 2	N 2	10	10	47.00	4.71	0.64	86.31	0.31	2.26	0.30	2.12	1.60	11.70
II	D1 or -A	N 2	N 2	4	11	47.92	4.90	0.68	86.32	0.30	2.19	0.32	2.34	1.65	12.06
III	D1 or -A	D1	-A	12	8	35.06	4.64	0.66	85.97	0.35	2.49	0.35	2.49	1.73	12.33
IV	N 2	D1	D1 or -A	4	8	37.82	4.70	0.66	86.24	0.31	2.25	0.30	2.18	1.65	11.09

* The figures in these three columns represent the weights of the young after removal of stomach and intestines.

† The figures in this column are given to the nearest whole number.

The results of our experiments can be summarised as follows (see also Tables I and II):

1. The diet of the father before conception had no influence whatever over the young, either on their number, weight or composition, at birth.

2. The diet of the mother before conception and during pregnancy has influenced the litter in some respects: the mothers kept on normal diet containing an excess of fat-soluble factor and calcium bore litters larger in number

and total weight than those born of mothers fed on usual or fat-soluble-deficient diets.

3. The larger total weight of the litters was due to the greater number of the offspring in each litter and not to the increased weight of each individual.

4. A large number of young were born dead in those litters, whose mothers were fed on deficient diets: in 4 litters from well-fed mothers only in one litter was one young rat found dead; in 16 litters from the mothers kept on a deficient diet, 1, 3, 5 and 7 dead young respectively were found in 4 litters.

5. At the day of birth the H_2O , Ca, P and N content in the young was nearly the same irrespective of the mothers' previous diet. A slightly better composition of young was found in some of those (see Group III in Table II) from parents on the deficient diets.

6. The striking influence previously shown by Korenchevsky [1921, 1922], Korenchevsky and Carr [1923] of different diets of mother rats shown in the skeleton of their young 24–85 days after birth cannot therefore be due to a variation in the reserve of P and Ca in the foetuses and is probably due to the different degree of storage of fat-soluble factor in the foetuses.

7. The normal chemical composition shown to exist in foetuses born of mothers kept on a diet deficient in fat-soluble factor is in accord with the accepted belief that the maternal organism will as far as possible yield all the necessary substances to her offspring even by the sacrifice of her own tissues. However, in our experiments, the storage of these substances, though being sufficient at birth, did not suffice in the post-natal life of the offspring.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

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LXXVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART IV. THE HALOGEN PHENOLS.

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(Received July 6th, 1923.)

THERE are in constant use a large number of disinfectants which have proved of great service for purposes of external disinfection under various circumstances, but so far the parallel problem of internal disinfection has received no adequate solution. For success in this direction a wider knowledge would appear to be necessary on the following subjects:

1. The evaluation of the effects of introducing specific groupings in various positions in organic compounds on their physiological and germicidal activity.
2. The constitution of proteins and other essential constituents of living cells and the chemical and physical action of poisons thereon.

A certain amount of research has been conducted in each of these branches, perhaps most particularly in the case of the first. Among other groups the introduction of halogens in various organic compounds was investigated by Erlich and Bechhold [1906], and the general conclusion that the introduction of halogens increased bactericidal power was reached. The present investigations, while not directly probing the intricacies of protoplasmic composition, yet have a bearing on the above problems, and information concerning the following questions has been obtained:

1. The effects on germicidal power of the introduction of halogens in different positions in the aromatic nucleus and side chains.
2. The mechanism of disinfection in the case of the phenol and chloro-phenol series, as revealed by the correlation of their germicidal efficacies with their action on various proteins and other protoplasmic constituents.
3. The possibility of the discovery of substances that would find application as internal disinfectants.

HALOGEN PHENOLS.

In the first place, the bactericidal powers upon different micro-organisms of the *isomeric chlorophenols*, and certain other halogen-phenols, have been compared.

Germicidal power was determined by two methods.

1. Chick-Martin [1908] method (in absence of organic matter).
2. Inhibitory method in peptone-broth [Morgan, Cooper and Burt, 1923].

Table I. *Chick-Martin Method.*Phenol coefficients at 20°. Period of disinfection 30 minutes
Chlorophenols

Organisms	Chlorophenols			Bromophenol
	<i>o</i>	<i>m</i>	<i>p</i>	<i>p</i>
<i>B. coli</i> comm. (1)	3.4	5.2	5.0	7.4
" " (2)	4.1	5.6	—	—
<i>B. typhosus</i>	3.7	4.8	4.7	8.0
<i>B. pyocyaneus</i>	3.6	—	—	—
<i>Staph. py. aur.</i>	4.2	5.0	5.0	—
<i>Strept. haem.</i>	3.0	4.6	3.8	—

The results show that:

1. *m*-Chlorophenol is slightly more efficacious than *p*-chlorophenol, which in turn is more active than the *o*-isomeride. Position isomerism is thus an important factor determining bactericidal power.

2. Slight differences are observed in the relative bactericidal powers of the isomeric chlorophenols when tested with different organisms, but the selective action is not very marked.

3. *p*-Bromophenol is more effective than *p*-chlorophenol.

By the inhibitory test (48 hours at 37° in peptone-broth) coefficients of about the same value were obtained, indicating that the relative germicidal powers of phenol and its halogen derivatives were not appreciably affected by presence of organic matter, rise in temperature, or longer period of disinfection.

Tests by this method in nutrient agar media were also made with the acid-fast organism *Smegma*, and, as before, *p*-chlorophenol was more potent than the *o*-compound.

HALOGEN QUINOLS.

It was found that the solubilities of the series of chloroquinols were such as might afford a unique series for investigating the effect of continued halogen substitution, especially as the second hydroxyl group in *para*-position to the hydroxyl in phenol does not measurably affect germicidal action (Table II).

The tri- and tetra-bromoquinols, however, were insufficiently soluble, so this series is restricted to the mono- and di-bromo-compounds.

The bactericidal powers of the various quinols are given in the following table:

Table II.

Quinol	Phenol coefficients		
	<i>B. coli</i>	<i>B. typhosus</i>	<i>Staph. py. aur.</i>
Quinol	0.96	1.0	1.1
Mono-chloroquinol	5.8	6.0	5.2
Di-chloroquinol	12.7	12.6	—
Tri-chloroquinol	23.0	24.0	—
Tetra-chloroquinol	34.0	35.0	—
Mono-bromoquinol	11.4	—	—
Di-bromoquinol	13.8	—	—

It is seen that all the halogen quinols are much more active than either quinol or phenol, and that germicidal power invariably increases as substitution

of halogen continues. The entrance of the first halogen atom, however, has the greatest effect, and the increment in bactericidal power gradually becomes less with further substitution. As in the case of the phenol derivatives, the bromoquinols are more efficacious than the chloro-compounds, but the germicidal power of the bromo-derivatives appears more readily to approach a limiting value, with the result that dibromoquinol is only slightly more active than dichloroquinol. It is unfortunate that the tri- and tetrabromoquinols are insoluble.

Monochloroquinol may be regarded as *o*- or *m*-chlorophenol, in which a second hydroxyl group has been substituted. As a general rule the introduction of *OH* groups into organic substances tends to diminish germicidal power, but in the case of chloroquinol there is an exception to this generalisation, as it is actually more efficacious than either *o*- or *m*-chlorophenol.

It was previously found that the bactericidal and inhibitory powers of the chlorophenols were much of the same order. The following results, however, show that this is not the case with the chloroquinols.

Table III.

	Inhibitory phenol coefficients <i>B. coli</i> , 48 hours at 37°
Di-chloroquinol	10
Tri-chloroquinol	7
Tetra-chloroquinol	6

A comparison with Table II shows that the inhibitory coefficients are less than the germicidal figures, and furthermore, that while the latter increase with the number of halogen atoms, the former diminish in magnitude. It is significant that the chloroquinols caused the production of red colorations in the peptone broth, and it would appear that they became oxidised to chloroquinones, which were then put out of action by the peptone and other nitrogenous constituents.

In marked contrast are the results obtained with *p*-nitrosophenol and allied compounds, the bactericidal and inhibitory powers of which are set out in Table IV:

Table IV

Organism: <i>B. coli</i>		
Substance	Phenol coefficient 30 mins. 20°	Inhibitory phenol coefficient 48 hrs. 37°
<i>p</i> -Chlorophenol	5.0	5.0
<i>p</i> -Nitrosophenol	1.1	30.0
Aniline	0.5	0.5
<i>p</i> -Nitrosoaniline	—	340
<i>p</i> -Nitrosodimethylaniline	50	540

It is seen that while *p*-nitrosophenol is approximately equal to phenol and less efficacious than *p*-chlorophenol in actual germicidal power, yet it is considerably more active than either as an inhibitory agent. *p*-Nitrosophenol thus not only retains its activity in peptone broth, but becomes much more

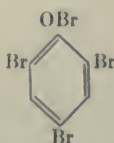
effective when the period of disinfection is lengthened and temperature raised to 37°. This is also the case with *p*-nitrosodimethylaniline, which is actually inhibitory in a concentration of 1 in 300,000. It is remarkable, furthermore, that while aniline is weaker than phenol as an inhibitor, *p*-nitrosoaniline is ten times more efficacious than *p*-nitrosophenol, and the substitution of methyl groups in the amino-group leads to a further enhancement in activity. The inhibitory power of these nitroso-compounds is destroyed by the presence of either serum or urine, so that it is unlikely that they will be of any value as internal disinfectants. Theoretically, however, the study of these compounds is of great interest, as aqueous solutions of nitrosoaniline and its dimethyl-derivative are decolorised by proteins, *e.g.* gelatin or egg-albumin, while with urine they give a brown coloration, and they thus seem to react chemically with proteins and allied substances.

PRACTICAL APPLICATION OF THE HALOGEN PHENOLS.

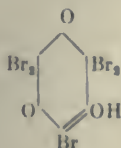
Erlich and Bechhold [1906] found there was insufficient margin between the bactericidal and toxic doses of the chlorophenols to enable these substances to be used for purposes of inner disinfection. Furthermore, they are known to be unstable in the animal organism, being converted into inactive sulphuric and glycuronic acid derivatives.

We therefore thought that it would be of practical interest to attempt to prepare derivatives of the phenols which are stabilised by the substitution of the hydrogen of the hydroxyl group. Experiments were therefore devised to replace the hydroxylic hydrogen by halogen.

Tribromophenol bromide



was first prepared by the action of excess of bromine upon phenol, and dichlorobromophenol bromide was prepared by the similar action of bromine upon the corresponding halogen phenol. These compounds are insoluble in alkali, liberate iodine from KI, and are probably tautomeric. They were, however, too insoluble in water to be of value as germicides, saturated solutions having no perceptible inhibitory power. A more promising water-soluble substance was next prepared by the action of bromine on phloroglucinol, *viz.* penta-bromodiketoxycyclo-hexenol.



The phenol-coefficient by the Chick-Martin method was 50 (*B. coli*), but, owing to the reactivity of certain of the bromine atoms, the substance failed in the presence of organic matter, and its inhibitory coefficient was < 1.5 with *Staphylococcus* and *B. typhosus*.

THE ACTION OF HALOGEN PHENOLS ON PROTEINS.

Although the chemical nature of the cell constituents of even the most familiar bacteria has only been investigated in an incomplete and fragmentary way, it is generally believed that their life functions are intimately associated with their protein content. It is therefore not illogical to suppose that information concerning the action of some chemical substances, having well pronounced germicidal properties, upon a variety of well-known proteins, might throw light upon the mechanism of the disinfecting process in cases where hitherto an explanation on a basis of chemical interaction, such as is possible with formaldehyde or sulphur dioxide, has not been regarded as probable. The best known of these so-called "non-chemical" germicides is phenol itself, which causes a very distinctive change in the nature of many proteins. Indeed the coagulating action of alcohol and phenolic compounds has been connected with their disinfectant rôle for some years. Moreover, analogies between coagulation produced by heat and by alcohol have been drawn by many workers; e.g. Spiro [1904], Pauli and Handuski [1909], Lewis and Mellanby [1910] and Lepeschkin [1923] are among contributors to our knowledge of this subject.

From experiments with phenol and *m*-cresol on gelatin, egg-albumin, egg-globulin, etc. [Cooper, 1912], the following conclusions were drawn as to the rôle played by these substances as germicidal agents.

1. The absorption of phenols by bacteria is only the initial stage in the process of disinfection.
2. The germicidal action which follows the absorption does not seem to be the result of a chemical union between the phenols and the bacterial proteins, but is apparently connected with the de-emulsification of the colloidal suspension.
3. The germicidal action appears to be similar in mechanism to that of heat.
4. The superiority of *m*-cresol to phenol as a germicide appears to be due to the fact that cresol precipitates proteins in lower concentrations than phenol.

By accurate analyses of phenol and *m*-cresol solutions after standing for suitable periods in contact with proteins in a manner described, figures for the partition of the disinfectants between the water and the protein phases were obtained. When certain concentrations of phenols were reached in the aqueous solutions of the disinfectants containing emulsified protein, the latter was precipitated and a low partition-coefficient $\left(\frac{\text{wt. of germicide in 1 g. protein}}{\text{wt. of germicide of 1 g. water}} \right)$

of about 3.0 was exchanged for a higher figure of about 10.0, which rose as the concentrations of the initial solutions were increased.

Thus in the curves of emulsoids showing the percentages of phenol in the protein plotted against the final concentrations in the water phase an initial straight line came to a termination at about 1.0 % of phenol in the case of egg-albumin, and at higher concentrations was continued at a much greater slope. The inversion point denoted the percentage of phenol which produced the changed condition in the protein, but no explanation was obtained as to the inner mechanism or the significance of the change in partition-coefficient.

The following experiments dealing with the action of phenol, *ortho*-, *meta*- and *para*-chlorophenols upon proteins, were designed to continue the above researches, and to connect quantitatively as far as possible the germicidal and physico-chemical evidence. In this way it was hoped to elucidate the problems raised in the work alluded to, and to correlate the structure of the above compounds, with their action on proteins and bacteria, and thus to explain the well-known specific effect of combined halogen of increasing germicidal power.

In many cases the results with phenol recorded in the papers cited have been verified, and these confirmatory experiments are included in the following for the sake of comparison with the analogous chlorophenol experiments.

PRELIMINARY TESTS.

Gelatin was submitted to the action of solutions of phenol and the three chlorophenols. With phenol no effect was noticed below 2.6 %, but in the case of the halogen compounds, distinct opalescence was discernible in concentrations as low as 0.4 %. In the case of egg-albumin, while 0.5 % phenol was necessary to cause a turbidity, 0.1 % solutions of the chlorophenols were sufficient to induce this change. This showed clearly that the chlorophenols were much stronger precipitants than phenol itself. The experiments also indicated that egg-albumin was more sensitive to the action of chlorophenols than gelatin, as had previously been shown in the case of phenol [Cooper, 1912].

This technique was not sufficiently precise for a quantitative comparison, so that further refinements indicated below were next introduced.

QUANTITATIVE COMPARISON OF THE PRECIPITATING ACTION OF PHENOL AND CHLOROPHENOLS ON EGG-ALBUMIN AND EDESTIN.

In the experiments described in this section, the action of the phenols was allowed to take place at the iso-electric point, and the minimum concentrations required to bring about complete precipitation were ascertained.

Solutions of egg-albumin were treated with the requisite volumes of hydrochloric acid, and phenol solutions. After standing 1 hour, the solutions were filtered from the coagulum and the filtrates tested for protein. In this way

the point of complete precipitation could be ascertained, and the following results were obtained:

Table V.

Concentration of egg-albumin		0.33 %
Acid required to adjust to iso-electric point		N/1245 HCl
Total volume of solutions		15 cc.
Minimum concentrations inducing complete precipitation.		
	%	Ratios
Phenol	1.45	1
<i>o</i> -Chlorophenol	0.57	2.5
<i>m</i> -Chlorophenol	0.34	4.3
<i>p</i> -Chlorophenol	0.34	4.3

It is thus seen that *m*- and *p*-chlorophenols are much more efficacious as protein-precipitants than *o*-chlorophenol, which in turn is more powerful than phenol. It would appear that the *m*-isomeride is rather more efficient than the *p*-compound, as the protein precipitate was always more definitely clumped in the former case.

Some similar experiments were next carried out with edestin. This protein was employed in the form of a solution in 7 % sodium chloride.

Table VI.

Minimum concentrations inducing complete precipitation.		
	%	Ratios
Phenol	1.15	1
<i>o</i> -Chlorophenol	0.22	5.2
<i>p</i> -Chlorophenol	0.21	5.5

In the case of edestin it is thus seen that *o*-chlorophenol is still more active as a precipitant than phenol, and closely approaches *p*-chlorophenol in precipitating power. A comparison of the above results with the bacteriological data in Table I indicates an interesting parallel between protein-precipitating and bactericidal power. It is seen also that an isomeride not only exercises a selective action on a particular micro-organism, but may also act selectively as a precipitant upon an individual protein.

DETERMINATION OF PARTITION-COEFFICIENTS OF PHENOL AND CHLOROPHENOLS BETWEEN WATER AND PROTEINS.

These estimations were carried out to ascertain to what extent the bactericidal and protein-precipitating powers of the above phenols were determined by their relative solubilities in water and proteins. The previous work had shown that, while the partition-coefficients for phenol and *m*-cresol in the case of emulsoid proteins were almost identical, *m*-cresol was distinctly a more powerful precipitant and germicide. In view of these results it was of great interest to extend the investigations to the chlorophenols. In the first place it was necessary to work out an accurate method for estimating chlorophenols, and for this purpose Lloyd's method [1905] was used. Preliminary experiments showed that with *o*- and *p*-chlorophenol accurate results could be obtained, two bromine atoms being quantitatively introduced in each case. *m*-Chlorophenol could not be accurately determined by this method, and thus the par-

tition-coefficients of only the *o*- and *p*-compounds have been studied. In the case of the cresols, however, it is interesting to note that it was the *m*-isomere alone that was amenable to accurate estimation [Cooper, 1912].

The partition-coefficients were determined by methods already described [Cooper, 1912], gelatin, emulsoid egg-albumin, and coagula being employed. In the case of the experiments with the emulsoids, a dialyser was employed to permit of the analysis of a protein-free water-phase.

1. Gelatin.

(a) The previous experimental work was first repeated and confirmed in the case of phenol.

Table VII.

Concentrations of phenol in water-phase	Partition-coefficient
	$\frac{\text{Gelatin}}{\text{Water}}$
0.4-2.6 %	3.0 (average)
2.6-4.0 %	5.0-15.8
	(gradually ascending with increasing concentration)

The change in partition-coefficient at 2.6 % was associated with a precipitation of the protein. Reversibility was complete, as by dilution of the water-phase the protein assumed its normal appearance and the coefficient returned to 3.

(b) Similar experiments were then carried out with *o*- and *p*-chlorophenol, and the results are as follows:

Table VIII.

Initial concentration in water-phase %	Condition of gelatin	Partition- coefficient
	1. <i>o</i> -Chlorophenol	
0.2-0.4	Normal	3.2
0.7-1.0	Partly precipitated	5-7
1.1-2.0	Complete precipitation	10-21
	2. <i>p</i> -Chlorophenol	
0.2-0.8	Normal	3.3
1.0	Partly precipitated	8.0
1.5-2.0	Complete precipitation	14-23

2. Egg-Albumin; Emulsoid Condition.

Table IX.

	Initial concentration in water-phase %	Condition of protein	Partition- coefficient
1. Phenol	0.1 -0.5	Normal	3.1
	1.0 -5.0	Precipitated	8.7-13.9
2. <i>o</i> -Chlorophenol	0.25	Normal	3.5
	0.50	Slight precipitation	4.3
	0.75-1.0	Partial "	6.7-13.5
	1.5 -2.0	Complete "	15.0-29.1
3. <i>p</i> -Chlorophenol	0.20-0.31	Normal	5.0
	0.47	Partial precipitation	6.1
	0.75-1.8	Complete "	11.7-29.5

From these experiments the following conclusions have been drawn:

1. The partition-coefficients for phenol and the chlorophenols in the case of normal gelatin and emulsoid egg-albumin are with one exception of the same magnitude, 3.0 approx. Only in the case of *p*-chlorophenol with egg-albumin is the figure somewhat higher, 5.0.

The coefficient for *m*-cresol was also found to be of the order of 3 [Cooper, 1912].

It is thus seen that in the case of various phenols with entirely different protein-precipitating and bactericidal powers, the relative solubilities in proteins and water are of the same order of magnitude, similar results being obtained with proteins widely divergent in character. These results confirm the original conclusion that the solubility of the phenols in proteins does not account for the differences observed in the precipitating and germicidal efficacies.

2. Coagulation of the proteins by the various phenols is invariably accompanied by a very considerable increase in the value of the distribution-coefficients, and this change is induced at different concentrations according to the precipitating power of the phenol.

3. The precipitation of gelatin is in every case reversible, and the reversion is accompanied by a return to the normal partition-coefficient.

4. The precipitation of egg-albumin is always irreversible, and the high coefficients are maintained after dilution of the water-phase.

3. *Egg-Albumin; Heat Coagulum.*

Table X.

	Phenol concentrations %	Partition-coefficients
Phenol	0.1-4.3	10.0
<i>o</i> -Chlorophenol	0.1-2.5	28.8
<i>p</i> -Chlorophenol	0.1-2.5	36.0

In all three cases, the protein being in the same condition throughout, the partition-law was strictly complied with, the amount of phenol dissolved in the protein being always directly proportional to the concentration. The coefficients were also all consistently higher than those obtained with the emulsoid protein.

It is of interest, furthermore, to note that, while with the gel and emulsoid proteins, the partition-coefficients bore no relation to the protein-precipitating and bactericidal powers, in the case of heat-coagula there is a distinct correspondence:

	Partition-coefficients (From Table X)	Precipitating ratios (From Table V)	Bactericidal phenol coefficients (average) (From Table I)
Phenol	1.0	1.0	1.0
<i>o</i> -Chlorophenol	2.9	2.5	3.6
<i>p</i> -Chlorophenol	3.6	4.3	4.6

It is not possible to say whether this correspondence is accidental, or is of some physico-chemical significance.

These results show that the partition-coefficient can be increased through coagulation either by addition of a minute amount of sodium citrate, or by adjustment to the iso-electric point by acid (the amounts of electrolytes used in each case having been found to have no effect on the coefficient for normal protein).

The salting out experiment is of special interest, because in this case a large water-transfer is induced without chemical change. When albumin is treated with a saturated solution of NaCl, water passes from the protein to the continuous phase, but the partially dehydrated protein does not separate in particulate form until the reaction is adjusted to the iso-electric point. It was previously shown [Reichel, 1909] that the addition of salt increases the absorption of phenol by heat-coagulated proteins through a physical disturbance in the solubility-ratio, and it was therefore important to control this experiment very carefully.

The results show that saturated salt solution only increases the coefficient from 2.9 to 7.2, but by adjustment to the iso-electric point there is a further rise to 19.8. As the presence of salt increases the solubility of phenol in coagula (Reichel) where water-transfer is absent, it is clear that the increased partition-coefficient is associated with the electrical adjustment rather than with the act of water-transfer or "salting-out."

A consideration of all the results in fact points to the conclusion that the increase in coefficient-value has an electrical explanation, *i.e.* is associated with the discharge of protein particles at the iso-electric point, and both sets of partition-coefficients appear to be realities.

It is noteworthy that the partition-coefficients do not change as the result of denaturation effected either by heat or phenol. It would thus appear that the increased uptake of phenol at precipitation is not of biological significance, but is a sequence to denaturation only induced by the iso-electric condition. Further support of this view has been obtained by some imbibition experiments in which use was made of the well-known fact that in the presence of small concentrations of acid, gelatin swells to an amount greatly exceeding its usual extent in water, and takes up a corresponding large volume of water into the gel structure.

In these experiments the distribution of phenol between gelatin and water was investigated under three different conditions:

1. In the ordinary way.
2. In the presence of $N/20$ HCl.
3. The gelatin was first dissolved in warm water and allowed to set. The volume of water was then made up to that used in 1 and 2 by the addition of a phenol solution, such as to yield the same initial concentration.

The results were as follows:

Table XII.

Exp.	Phenol	Gelatin	Partition-coefficient
1	50 cc. 0.8 %	1 g.	2.9
2	"	"	3.2
3	"	"	3.4

Thus the actual distribution of the water did not measurably affect the partition-coefficient.

THE NATURE OF THE ACTION OF PHENOLS ON PROTEINS.

So far it is only known that phenols act in some way upon proteins to cause their coagulation at the iso-electric point. Experiments have therefore been devised to prove finally that phenols induce some chemical change analogous to heat-denaturation, and are not merely protein-precipitants causing coagulation under favourable conditions.

To solutions of egg-albumin were added solutions of phenol, alcohol, *m*-cresol, *o*-, *m*-, and *p*-chlorophenol, and the mixtures were dialysed until the odour of the reagents had disappeared. It was then found that on adjusting the reaction to the iso-electric point, the protein was coagulated. This shows clearly that the phenols (and also alcohol) react in some unknown way with proteins, with the result that the colloidal condition of the latter is permanently altered, and separation in particulate form will take place by discharge even after removal of the reagents. Furthermore, as showing that the protein-precipitating power of the phenols is merely an expression of their "denaturing" action, it was found that chlorophenols and cresol could effect this primary change in lower concentration than phenol itself.

It would seem that the denaturation induced by phenol is not identical with that brought about by heat. Thus, during heat coagulation, sulphuretted hydrogen is evolved, and can be detected either by lead acetate or sodium nitroprusside [Harris, 1923], but we find that these tests give negative results in the case of coagulation by phenol.

The exact nature of denaturation is at present unknown, but there is evidently a distinct parallel between denaturing and bactericidal action in the case of phenols and their halogen derivatives, the introduction of a halogen atom into phenol considerably increasing both these activities, and to approximately the same degree.

The germicidal action may be due not to an actual separation of the cell-proteins in particulate form, but to an enlargement of the colloidal units as a result of denaturation and transfer of water to the protein-phase. As a consequence, the surface area of the disperse-phase is diminished, and sufficient retardation in metabolism may take place to cause finally the death of the organism.

THE DISTRIBUTION OF PHENOL BETWEEN LECITHIN AND WATER.

It was deemed of interest to ascertain if other colloids would take up phenol analogously to the proteins studied. A solution of "dialysed iron" was found to be too weak to be useful in such work, but lecithin, which forms an emulsoid solution, was found to absorb phenol in measurable quantity. The equilibria were determined as in the case of the albumin emulsoid, 20 cc. of 2 % lecithin solution being used inside the dialyser, and 50 cc. phenol solution outside.

With strong phenol solutions acting for 24–48 hours, the lecithin emulsion was changed to a thick, oily sediment, and weaker solutions effected a partial precipitation from the true emulsoid state. Normally, the lecithin formed a stable emulsion over these periods. The coefficients and other data are given in Table XIII. The absorption coefficients appear to rise gradually with the increase in concentration and progress of the agglutination of the lipid particles, as in the case of proteins.

Table XIII. *Lecithin and Phenol.*

20 cc. 2 % lecithin solution Phenol. Final concentration water-phase	50 cc. phenol solution Distribution coefficient
0.2726 %	5.1
0.2949	8.2
1.319	10.4
2.093	14.4

These results are of interest in connection with the work of Schiemann and Ishiwara [1914], who showed that the presence of lecithin diminished the bactericidal power of phenol. Evidently the phenol is readily taken up by lecithin and less is therefore available for disinfection. It is also possible that this alteration in the condition of the lecithin emulsion is associated with bactericidal action quite as much as the changes induced in emulsoid proteins, and, although it is true that proteins are fundamental constituents of living cells, yet the death of the organism may be due in reality to a "denaturation" of the whole colloidal complex of the cell.

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LXXVIII. ADSORPTION AND MECHANISM OF POISONING.

PART I. IRRITANT POISONS.

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IN order that we may evaluate the effect produced upon life functions by the substances artificially ingested into the organism it is necessary, according to our present ideas, to postulate that the substances enter into some sort of a combination with the constituents of the cells, which in turn transmit the effect to the whole organism. It is evident that such an interaction depends not only upon the structure of the ingested reagent, but quite as much upon the structure of the cell-substances. From the chemical standpoint great difficulties arise in an attempt to study the effect of poisons directly upon higher organisms, because of the numerous disturbing influences, such as physiological activities and localisation of poisons in particular regions of the animal system, etc. Therefore, an attempt has been made in the present work to simplify matters as far as possible by choosing comparatively simple substances for purposes of experimentation. Of course the results thus obtained are by no means absolute, yet they throw some light on this difficult problem and they may possibly indicate the probable trend of reactions in higher organisms, under a similar set of conditions.

Moore and Roaf [1904, 1906] have shown that chloroform and other anaesthetics combine with protoplasm to form unstable compounds and they presume that the formation of such compounds is the cause of intoxication. The close relationship between intoxication and poisoning can justify our holding the above view with regard to the mechanism of poisoning also. Again, Loew [1893] assumes a direct chemical union of the protoplasm with the so-called "substituting poisons," supposing the labile amino and aldehyde groups of the protoplasm to be active in effecting such a union. Such an explanation seems to be, at least in some instances, quite plausible. For example, hydroxylamine and the hydrazines, which are well-known aldehyde-reagents, are powerful poisons, while the ketoximes in which the reaction group is bound, are not so poisonous. Among others, the work of P. Karrer [1918] on the action of toxins, may be specially mentioned, as supporting Loew's theory of chemical reaction between poisons and the affected tissues. However, in spite of the support lent to this theory by numerous workers, it has failed to explain

satisfactorily the mechanism of poisoning in a very large number of cases in which it has been established on fairly firm grounds that no chemical action takes place, and that the effects produced are due to surface action only. Ehrlich, for example, maintains that during the course of his numerous experiments in this direction, he was never able to establish that there had been a chemical union between the poison and the affected part of the body. In fact, he found that the various poisons could be again extracted from the tissues by means of neutral chemically inactive solvents. However, Ehrlich [1909] himself mentions a number of toxins and poisons, *e.g.* cocaine, stovaine, and compounds of trivalent arsenic, etc., as being exceptions to his generalisations.

Thus it appears that at the present stage of our knowledge we cannot accept either Loew's or Ehrlich's theory to the entire exclusion of the other. On the other hand, we must accept both, for it appears that there are cases of simple chemical union, simple surface action, and cases involving both surface action and chemical action.

Howsoever this conflict of opinions regarding the mechanism of actions may be decided, at least it is certain that the production of an effect at any desired place in the organism necessitates such a constitution of the ingested substance as to make possible its local fixation, whether it be of a chemical or of a physical nature. In other words, "sorption" [McBain, 1919] of a poison by the tissues is necessary for the production of any physiological effects. The old notion that a poison may kill by its action through the nervous system without sorption, cannot be retained in the light of our present knowledge, and therefore a study of the sorption of poisons by the principal constituents of the body, is bound to be of some interest in the elucidation of the mechanism of poisoning.

Poisons belonging to the irritant type have been dealt with in the present work, and investigations on the other groups are in progress. By far the most important of the irritant poisons are salts of arsenic, lead, copper, antimony, and chromium, and these substances are characterised by their irritant effects upon the tissues, accompanied by well-marked nervous influences.

Apart from the chemical nature of the inorganic poisons the physical factors concerned in their solution, etc., are of great importance. The part played by such factors can be easily realised when it is considered that the specific effects of such poisons depend chiefly, if not exclusively, upon their constituting ions [Blake, 1839, 1881]. Quite in agreement with the assumption of ionic effect in these physiological studies, is the great importance of the degree of dissociation, which is very decidedly pointed out by the investigations of Dreser [1893] and Paul and Krönig [1896] upon the bactericidal action of mercury salts. A further support of this theory is provided by the comparative inactivity of cacodyl compounds as poisons (Crum Brown), in spite of their arsenic content and marked solubility. The inertness of such compounds is easily explained by the fact that the arsenic in them is bound up to an organic radical and is not present in the ionic state even in water.

Results obtained by Kahlenberg [1900] and Loeb [1897, 1898], during their investigations on the action of salts on bacteria, are also in general agreement with the ionic theory of poisoning.

On the basis of the view that sorption precedes poisoning and that poisoning, at least in the case of most inorganic salts, is ionic in nature, the present work was carried out with the object of determining the sorption of the various poisons in the different regions of the animal system with a view to elucidate the mechanism of poisoning.

EXPERIMENTAL.

Caseinogen, representing the class of protein substances which form the chief constituents of the animal body, was chosen as the adsorbent in the course of the present work, and a number of experiments were also performed with saliva, bile, and blood-serum. The poisons tried were lead acetate, arsenic oxide, and copper sulphate.

The general procedure followed in all the experiments is outlined below.

Aqueous solutions of each poison in six different concentrations were prepared. Equal volumes (50 cc.) of each of these solutions were pipetted out into dry stoppered bottles and then a weighed amount (1.0 g.) of caseinogen (the size of the particles being between 16–20 mesh) was introduced into each bottle. After thorough mixing the bottles were kept in a thermostat at 37° (body temperature) for a given time, after which the contents of each bottle were filtered separately through filter papers which were previously soaked with the particular poison in question, washed and dried. The amount of the poison left after sorption, was determined analytically in the filtrate and knowing the initial concentration of the poison used, the sorption of each poison per gram of the adsorbent (X/M) was calculated. The concentration of the poison in 1 cc. of each solution after sorption (volume concentration) was also determined and these results were plotted against the corresponding X/M . The results obtained with each poison are given below.

Lead Acetate.

Aqueous solutions of pure lead acetate of concentrations approximating to $N/10$, $N/15$, $N/20$, $N/30$, $N/50$ and $N/100$ were prepared and stocked after filtration. 50 cc. of the poison of each concentration was used for each experiment and the extent of sorption was determined after one, three and six hours. Lead was estimated gravimetrically as lead chromate and the results, calculated in terms of $Pb(CH_3COO)_2$, are given in Table I.

The curves drawn from the data are shown in Fig. 1. The shape of these curves indicates clearly that they are typical "Adsorption Isotherms." Further support is lent to this view by the fact that logarithms of X/M and volume concentration when plotted, produce a straight line which is so characteristic of adsorption reactions.

Table I.

Concentration of original solution (in terms of $\text{Pb}(\text{CH}_3\text{COO})_2$ per litre)	After sorption for one hour		After sorption for three hours		After sorption for six hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
15.916 g	0.1105	0.01370	0.1464	0.01299	0.1482	0.01295
10.486	0.0824	0.00884	0.1196	0.00809	0.1263	0.00796
7.834	0.0711	0.00641	0.1121	0.00559	0.1225	0.00538
5.354	0.0694	0.00396	0.0966	0.00342	0.1121	0.00311
3.124	0.0448	0.00223	0.0596	0.00193	0.0655	0.00181
1.443	0.0242	0.00106	0.0376	0.00079	0.0351	0.00084

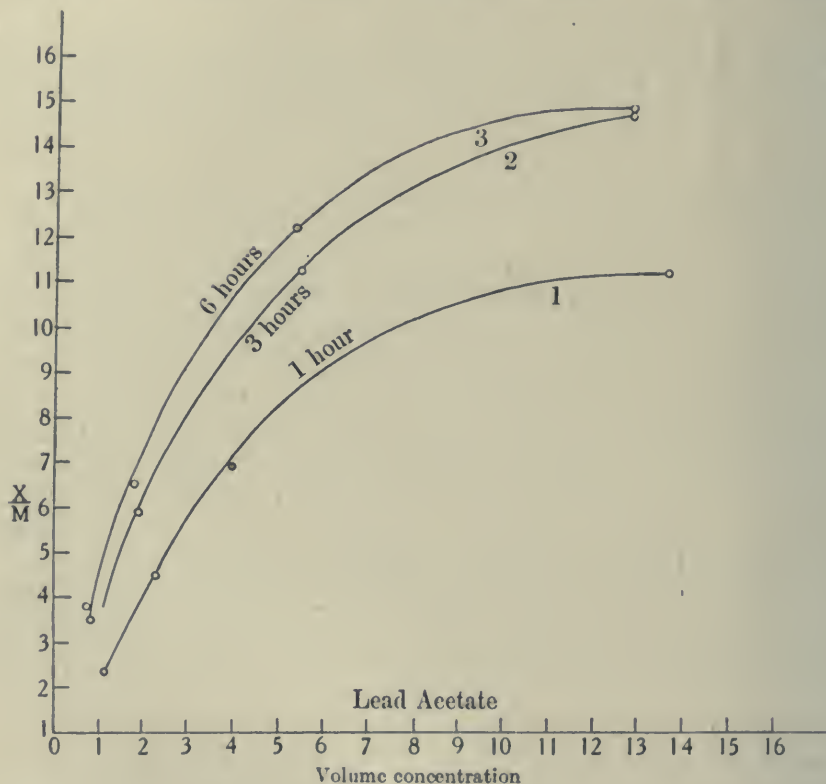


Fig. 1.

Arsenious Oxide.

Aqueous solutions of pure arsenious oxide of concentrations approximating to $N/15$, $N/20$, $N/30$, $N/50$ and $N/100$ were employed and the amount of arsenic in them was determined before and after adsorption, by titration against a standard iodine solution, in the presence of excess of sodium bicarbonate, using starch as an indicator. All other conditions of temperature, time, and procedure were the same as described before (p. 615). The results calculated, in terms of As_2O_3 are given in Table II.

Table II.

Concentration of original solution (in terms of As_2O_3 per litre)	After sorption for one hour		After sorption for three hours		After sorption for six hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
3.30 g.	0.00190	0.00326	0.00220	0.00325	0.00128	0.00327
2.477	0.00107	0.00245	0.00107	0.00245	0.00092	0.00246
1.65	0.00072	0.00163	0.00072	0.00163	0.00048	0.00164
0.9895	0.00027	0.00098	0.00027	0.00098	0.00002	0.00099
0.4949	0.00022	0.00049	0.00022	0.00049	0.00012	0.00049

The nature of the equilibrium after one and three hours is distinctly different from the usual adsorption equilibrium, and what is more striking, the equilibrium conditions in the last four concentrations appear to be the same for the first three hours, after which there is a marked change, whereby the nett results of sorption after six hours, are sufficiently influenced to be thrown into the form of an "Adsorption Isotherm."

Relation between Adsorption and the Degree of Dissociation of a Poison.

It has been clearly brought out by the investigations of Dreser, and of Paul and Krönig, that substances which are dissociated to a greater extent are more powerful disinfectants and antiseptics than others which are dissociated to a lesser degree. This and other similar observations led the author to investigate the relation between adsorption and the degree of dissociation of a poison in solution. With this end in view the equivalent conductivities of the poisons at various concentrations were determined at 37° , and these results were plotted against X/M of the corresponding solutions. The curves so obtained are shown in Fig. 2. It is evident that the curves for lead acetate and arsenious oxide are both hyperbolic, and that for each concentration of the poison

$$A(C - P) = \text{Constant},$$

where $A = X/M$,

C = Equivalent conductivity at 37° .

P = a constant depending upon the nature of the poison.

It also appears that poisoning is a function of the adsorption and the degree of dissociation of any particular poison. It is further clear from Fig. 2 that with increasing concentrations of the poison the adsorption (per gram of the adsorbent) increases, while the equivalent conductivity of the corresponding solution decreases and consequently the enhanced poisonous effect due to greater adsorption of the poison is partly neutralised by the lowering of the dissociation. These facts clearly point out that there is a particular concentration at which the combined poisonous effect due to adsorption and dissociation is the maximum.

Work in this direction is in progress and the results are expected to show that merely the quantity of a poison, independent of its concentration, is not a sufficient indication of the minimum fatal dose of that poison. It is expected that there will be one particular concentration for each poison at which the

lethal dose will be most effective. On the basis of such results it may also be possible to establish certain mathematical relationships between adsorption, concentration and the lethal dose of a poison.

Copper Sulphate.

Aqueous solutions of pure Copper sulphate, of concentrations approximating to $N/1$, $N/5$, $N/10$ and $N/100$ were employed and 40 cc. of the solution of each concentration were used for each experiment. The general procedure adopted during the course of these experiments was the same as described before (p. 615), with the exception that the experiments were conducted at 28° and the extent of sorption was determined after one-fourth, one and three hours. Copper sulphate was estimated volumetrically by determining the amount of iodine liberated by it from a solution of potassium iodide. A standard solution of sodium thiosulphate was used for estimating the iodine thus liberated.

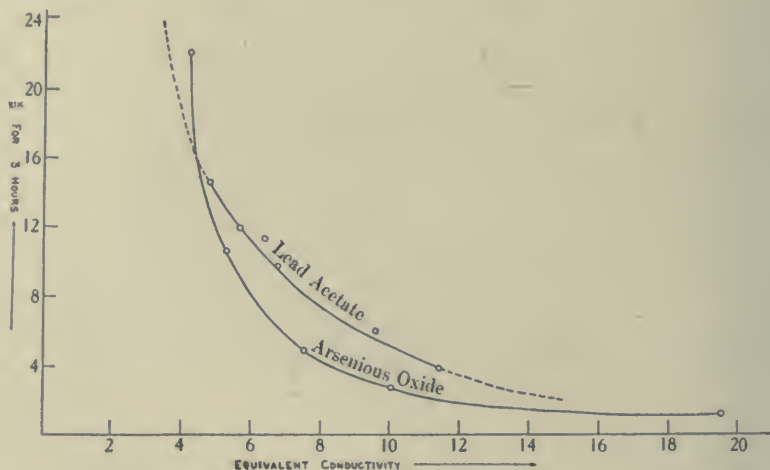


Fig. 2.

The results of the sorption of copper sulphate by caseinogen, calculated in terms of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ are given in Table III.

Table III.

Concentration of original solution (in terms of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per litre)	After sorption for one-fourth hour		After sorption for one hour		After sorption for three hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
122.7 g.	0.02873	0.1220	0.04357	0.1216	0.04696	0.1215
24.82	0.02572	0.0242	0.03803	0.0239	0.04473	0.0237
12.26	0.01823	0.0118	0.03202	0.0115	0.03702	0.0113
1.207	0.00549	0.0011	0.00859	0.0010	0.00875	0.0010

If curves are drawn from the above data they are seen to be typical "Adsorption Isotherms."

Copper Sulphate and Saliva.

A set of sorption experiments was conducted with approximately $N/1$, $N/5$, $N/10$ and $N/100$ solutions of copper sulphate, using human saliva as the sorbent. A few hours after meals, the salivary glands were excited by chewing some black pepper, and after thorough rinsing of the mouth with water, saliva was collected in a clean beaker. 5 cc. of this saliva were used for each experiment. All other conditions of experiment were the same as described before (p. 615). The extent of sorption of copper sulphate was determined after one-fourth, one, and twenty-four hours. The results, calculated in terms of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sorbed per gram of saliva, are given in Table IV.

Table IV.

Concentration of original solution (in terms of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per litre)	After sorption for one-fourth hour		After sorption for one hour		After sorption for twenty-four hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
123.492 g.	0.00505	0.12287	0.00505	0.12287	0.00505	0.12287
24.81	0.00493	0.02420	0.00493	0.02420	0.00523	0.02417
12.26	0.00395	0.01177	0.00417	0.01174	0.00417	0.01174
1.207	0.00017	0.00119	0.00088	0.00110	0.00088	0.00110

By plotting the data given in Table IV, typical adsorption curves are produced, even in the case of saliva.

While experimenting with saliva it was observed that a precipitate was formed immediately on adding saliva to a solution of copper sulphate. Later it was found that similar observations had been recorded before in the case of other albuminous substances (*Practical Biological Chemistry*, by G. Bertrand and P. Thomas). However, it was considered worth while to study the nature of the precipitates thus obtained in order to determine their composition and the probable mechanism of their formation. With this end in view, equal amounts of saliva were added to equal volumes of copper sulphate solutions of various concentrations, and the copper-content of the precipitates thus formed, was determined after one-fourth hour and twenty-four hours. These results show that the copper-content of these precipitates is almost the same after fifteen minutes of sorption as it is after contact with the copper sulphate solution for twenty-four hours.

The facts that a precipitate is formed immediately on adding saliva to a solution of copper sulphate and that the maximum sorption takes place almost instantaneously, point to the existence of some close union between saliva and copper sulphate. Again, the amount of copper sulphate adsorbed per gram of saliva was found to vary with the concentration of the copper sulphate solution used, indicating thereby the absence of a purely chemical combination between the two substances. The only other alternative is to assume the formation of certain chemico-adsorption-compounds by the interaction of copper sulphate solution and saliva. This view is further supported

by a fact which has been clearly brought out in this work, namely, that the principal reactions between aqueous copper sulphate and saliva bear a close analogy to the phenomenon of adsorption.

Sorption of Copper Sulphate by Blood-serum and Bile.

In one set of experiments serum prepared from fresh goat's blood was used as a sorbent for copper sulphate and another set of experiments was conducted in which fresh goat's bile was employed as the sorbent. Approximately $N/1$, $N/5$, $N/10$ and $N/30$ solutions of copper sulphate were used in both the cases. 5 cc. of blood-serum or bile were employed for each experiment. 40 cc. of copper sulphate solution of each concentration were used with blood-serum, while in the case of all experiments with bile, only 20 cc. of the poison were employed each time. All other conditions of experiment were identical with those described before (p. 615). Some of the results obtained in the case of blood-serum are given in Table V.

Table V.

Concentration of original solution (in terms of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per litre)	After sorption for one-fourth hour		After sorption for twenty-four hours	
	X/M	Volume concentration	X/M	Volume concentration
124.815 g.	0.00447	0.12425	0.00594	0.12406
24.963	0.00287	0.02460	0.00402	0.02445
12.4815	0.00173	0.01226	0.00411	0.01198
4.1605	0.00039	0.00367	0.00062	0.00337

Results very similar in nature to those given in Table V were obtained in the case of bile also. Curves plotted from these data reveal the fact that bile and blood-serum behave exactly like caseinogen and saliva in the matter of adsorbing copper sulphate from its aqueous solutions.

It may be mentioned here that the phenomenon of the formation of chemico-adsorption-compounds was observed in the case of blood-serum also—a fact which was verified in a manner similar to the one employed in the case of saliva.

CONCLUSIONS.

The results of this investigation show that adsorption is the most predominant feature in the interaction between poisons like lead acetate, arsenious oxide and copper sulphate and substances of the type of caseinogen and blood-serum, etc. In a number of cases, the formation of chemico-adsorption-compounds has also been pointed out. These conclusions naturally lead one to think that the more complex phenomenon of poisoning in the living animal is also probably governed, at least in its initial stages, by laws essentially similar to the ones that guide and control the ordinary laboratory reactions of the type indicated in this paper. If the results indicated here represent, even in some degree, the trend of action of such poisons in the living organism, it is clear that adsorption forms the first step in the very complicated process of poisoning due to irritant poisons. It cannot be said at this stage whether all other

poisons act in a manner similar to the one in which irritant poisons appear to behave. Further work is necessary in this direction and it is hoped that the extended investigation may throw some more light on this obscure yet extremely interesting phenomenon of the mechanism of poisoning.

My best thanks are due to Dr S. S. Bhatnagar, for his suggestion of the problem and for the keen interest he has taken in it throughout.

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LXXIX. THE SUPPLEMENTARY VALUE OF LIGHT RAYS TO A DIET GRADED IN ITS CONTENT OF FAT-SOLUBLE ORGANIC FACTOR.

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INTRODUCTION.

HUME [1922], and the authors [1922] showed that rats on a diet very deficient in the fat-soluble growth-promoting factor, if they are irradiated for about ten minutes daily by the mercury-vapour quartz lamp, grow much better than non-irradiated rats on the same diet. But they do not go on growing normally; eventually growth fails, and the subsequent history is that of non-irradiated rats on a — *A* diet. This led to the conclusion that light rays promote growth by enhancing and prolonging in some way the action of the fat-soluble *A* which is stored in the body of animals at the time they are put on the — *A* diet. Now some rats on the — *A* diet employed (diet *F* of this investigation) develop experimental rickets [Korenchevsky, 1921; Goldblatt, 1923]. But histological examination of the bones of many irradiated rats on the same — *A* diet revealed no evidence of rickets¹. Most of them were practically normal, the only pathological change observed in some being slight or moderate osteoporosis. Chemical examination of the bones of these irradiated rats showed a much higher percentage of calcium than in the control rats, but lower than that of normal rats on a complete diet¹. Recently one of the authors [Goldblatt, 1923] showed that when rats are fed on diets graded in their content of fat-soluble organic factor (from the optimal amount to practically complete absence of it) there results a corresponding gradation in the growth of the animals and in the degree of calcification of their bones. The present study was planned in order to determine the minimal amount of fat-soluble organic factor which, supplemented by light rays, can effect the normal growth of rats as well as the normal development and calcification of their bones.

¹ Unpublished.

EXPERIMENTAL.

In this investigation the same six diets were employed as in the study of the effect of grading the quantity of fat-soluble organic factor in a diet upon the development and calcification of the bones of rats [Goldblatt, 1923]. The basal diet, of which the six diets employed were variations, consisted of

Commercial casein	20
Starch (wheat)	50
Cotton-seed oil	15
Salt mixture (McCollum and Davis, No. 185)					5
Marmite	5
Lemon-juice	5
Distilled water	50

When the casein employed is the unpurified commercial product, it supplies to the above diet an amount of fat-soluble organic factor which will promote about 66 % of the growth of a rat on the same diet, but with sufficient cod-liver oil added to supply an optimal amount of the organic factor. The diet containing 20 % of unpurified casein as the sole source of fat-soluble organic factor will be referred to as diet *B*. An attempt was also made to determine the exact amount of fat-soluble growth-promoting factor in this diet. Rats were put on a diet completely deficient in fat-soluble *A* (diet *F* of this investigation), and when they had ceased growing for three weeks were changed to *B* diet and in addition varying amounts of cod-liver oil were administered daily. Of this oil, 2.25 mg. daily were known to promote practically normal growth for four weeks in a rat that had ceased growing on the —*A* diet (diet *F*). When *B* diet was substituted, the addition of only 0.56 mg. of oil a day was necessary to promote the same amount of growth in four weeks. Thus the fat-soluble *A* in the remaining 1.69 mg. must have been supplied by diet *B*. A closer titration was not attempted. Thus it would seem that diet *B* contains approximately 75 % of the amount of fat-soluble *A* necessary to promote normal growth. This agrees rather well with the previous statement that diet *B* is able to promote about two-thirds of the normal growth of rats during a period of eight weeks.

In the previous investigation 3 % of crude cod-liver oil was added to diet *B*, and this resulted in a diet which contained an amount of fat-soluble organic factor considerably in excess of the optimal. This diet was called *AA* and considered the normal control diet. In the present investigation, instead of incorporating the cod-liver oil with the diet, the normal control rats were kept on *B* diet and 200 mg. of crude cod-liver oil were administered by pipette daily to the rats. This will also be referred to as diet *AA*. This is approximately half the amount of cod-liver oil which the rats on diet *AA* in the former study received, but is also far in excess of the minimal amount necessary to promote normal growth. When tested by the biological method of Zilva and Miura

[1921] 2.25 mg. daily of the oil employed gave a positive result, *i.e.* was able to promote normal growth for four weeks.

By replacing various amounts of the unpurified casein in diet *B* by casein inactivated by heat and oxidation, the amount of fat-soluble organic factor was reduced. Diets *C*, *D*, *E* and *F* were thus made, which differed from *B* only in the quantity of organic factor they contained. The following table illustrates the source and quantity of the fat-soluble organic factor in the six diets.

Table I.

Diet	Basal diet containing		
	Unpurified casein	Purified casein	Cod-liver oil
<i>AA</i>	20	0	200 mg. daily
<i>B</i>	20	0	0
<i>C</i>	15	5	0
<i>D</i>	10	10	0
<i>E</i>	5	15	0
<i>F</i>	0	20	0

Thus, considering that diet *B*, in its 20 % of unpurified casein, contains about 70 % of the minimal amount of fat-soluble *A* which will promote normal growth, it follows that diet *C* contained 52.5 %, *D* 35 %, *E* 17.5 % and *F* none at all.

Forty-eight rats (four litters of twelve¹) were employed in this investigation. From every litter two rats were put on every one of the six diets, one being irradiated for ten minutes daily at a distance of 60 cm. by the mercury-vapour quartz lamp. Thus there were four rats on every one of the six diets (a total of twenty-four) which were irradiated daily, and the same number on the same diets who were kept in a well-ventilated room with a large double window of plate glass half an inch thick through which light had to filter. Except during the time the irradiated rats were under the lamp, they were kept in the same room as the non-irradiated controls.

Growth.

The average final weight and average maximum gain in weight of the non-irradiated rats on the six diets showed a gradation which corresponded roughly with the gradation in the fat-soluble organic factor content of the diet (see Table II). The non-irradiated rats on diet *AA* grew even slightly better than the irradiated rats on the same diet. There were two males in the former group and only one in the latter. That easily accounts for the slight difference, because males on a normal diet always grow better than females. But on the deficient diets *B*, *C*, *D*, *E* and *F* the non-irradiated rats did not grow nearly as well as the irradiated ones on the corresponding diets.

¹ The mothers of these litters were kept during pregnancy and lactation on a diet consisting of white bread, oats, maize, bran, cabbage leaf and winter milk. This diet is not very rich in fat-soluble organic factor.

Table II. *Non-irradiated Control Rats.*

No. of rat	No. of litter	Sex	Diet	Initial age days	Final age days	Days on diet	Initial weight grams	Final weight grams	Gain in weight grams	Average amount of food eaten daily grams	In bones			Histological diagnosis
											% H ₂ O	% Ca wet	% Ca dry	
G 596	XXIII	♀	A.A	22	78	56	29	130	101	11.6	42.35	11.67	20.81	Normal
G 597	XXIV	♂	A.A	22	78	56	19	137	118	11.6	45.93	11.28	20.86	
G 598	XXV	♂	A.A	21	77	56	30	165	135	12.4	41.60	11.54	19.65	
G 599	XXVI	♀	A.A	20	76	56	23	149	126	12.4	42.18	12.59	21.78	
Averages											43.01	11.77	20.77	
G 604	XXIII	♀	B	22	78	56	27	89	62	11.5	50.06	8.43	16.87	Moderate rickets and slight osteoporosis
G 605	XXIV	♂	B	22	78	56	29	126	97	11.5	40.24	9.96	18.53	
G 606	XXV	♂	B	21	77	56	36	116	80	12.8	47.00	9.35	17.63	
G 607	XXVI	♀	B	20	76	56	30	116	86	12.8	40.52	11.85	19.93	
Averages											45.95	9.89	18.24	Nearly normal
G 612	XXIII	♀	C	22	78	56	27	76	49	9.1	47.50	8.67	16.50	Very slight osteomalacia
G 613	XXIV	♂	C	22	78	56	28	74	46	9.1	48.83	7.26	14.18	
G 614	XXV	♂	C	21	77	56	36	93	57	10.1	47.53	9.23	17.53	
G 615	XXVI	♀	C	20	76	56	31	88	57	10.1	46.73	8.48	15.93	
Averages											47.64	8.41	16.03	
G 620	XXIII	♀	D	22	78	56	29	83	54	9.7	46.63	7.34	13.73	Slight rickets and slight osteoporosis
G 621	XXIV	♂	D	22	78	56	28	103	75	9.7	47.62	7.67	14.63	
G 622	XXV	♂	D	21	77	56	34	88	54	9.9	41.62	10.16	17.36	
G 623	XXVI	♂	D	20	76	56	36	112	76	9.9	48.50	9.25	17.87	
Averages											46.05	8.60	15.89	Very slight osteomalacia and severe osteoporosis
G 628	XXIII	♂	E	22	78	56	33	91	58	10.3	48.05	8.28	15.94	Very slight rickets and slight osteoporosis
G 629	XXIV	♂	E	22	78	56	33	68	35	10.3	46.90	7.73	14.57	
G 630	XXV	♂	E	21	77	56	30	63	33	8.5	45.51	8.77	16.11	
G 631	XXVI	♀	E	20	76	56	24	54	30	8.5	49.69	6.94	13.77	
Averages											47.53	7.93	15.09	Slight rickets
G 636	XXIII	♀	F	22	78	56	31	68	37	8.0	48.80	8.37	16.36	Slight rickets and moderate osteoporosis
G 637	XXIII	♂	F	22	78	56	36	51	15	8.0	44.85	8.13	14.72	
G 638	XXIV	♂	F	22	78	56	33	72	39	8.0	50.48	6.86	13.88	
G 639	XXV	♀	F	21	77	56	41	111	70	9.6	43.40	9.22	16.34	
G 640	XXVI	♀	F	20	76	56	28	55	27	9.6	45.73	7.50	13.84	Moderate rickets and slight osteoporosis
Averages											46.65	8.10	15.02	

The irradiated rats on diets *AA*, *B* and *C* grew equally well and reached normal weights for rats of that age and breeding. The average maximum gain in weight and average final weight of the irradiated rats on diet *D* were only slightly less (7.5 %) than those on diet *AA*. But the irradiated rats on diets *E* and *F*, although they grew very much better than the non-irradiated controls on the corresponding diets, did not grow as well as those on the other four diets. Towards the end of the experimental period the growth of the irradiated rats on diets *E* and *F* had practically stopped, while the irradiated rats on the other diets were still making regular weekly gains in weight (see Table III).

Table III. *Irradiated Rats.*

No. of rat	No. of litter	Sex	Diet	Initial age days	Final age days	Days on diet	Initial weight grams	Final weight grams	Gain in weight grams	Average amount of food eaten daily grams	In bones			Histological Diagnosis
											% H ₂ O	% Ca wet	% Ca dry	
G 600	XXIII	♀	AA	22	78	56	27	129	102	12.2	43.23	12.84	22.64	Normal
G 601	XXIV	♀	AA	22	78	56	22	128	106	12.2	40.37	12.53	21.02	"
G 602	XXV	♂	AA	21	77	56	31	168	137	11.1	43.13	11.77	20.72	"
G 603	XXVI	♀	AA	20	76	56	25	109	84	11.1	46.38	11.37	21.22	"
Averages							26	133.5	107	11.6	43.27	12.12	21.40	
G 608	XXIII	♀	B	22	78	56	29	126	97	12.8	39.58	12.45	20.61	Normal
G 609	XXIV	♀	B	22	78	56	28	139	111	12.8	43.83	11.41	20.32	"
G 610	XXV	♀	B	21	77	56	36	122	86	13.3	39.80	12.50	20.74	"
G 611	XXVI	♂	B	20	76	56	29	160	131	13.3	42.02	11.73	20.29	"
Averages							30.5	137	106	13.0	41.30	12.02	20.49	
G 616	XXIII	♀	C	22	78	56	30	122	92	13.3	37.52	12.82	20.51	Normal
G 617	XXIV	♀	C	22	78	56	29	139	110	13.3	40.74	11.34	19.16	"
G 618	XXV	♂	C	21	77	56	35	150	115	13.0	40.58	10.37	17.46	"
G 619	XXVI	♀	C	20	76	56	31	122	81	13.0	39.53	12.04	19.91	"
Averages							31	133	99	13.1	39.59	12.06	19.86	
G 624	XXIII	♀	D	22	78	56	30	108	78	13.1	39.79	13.12	21.79	Normal
G 625	XXIV	♂	D	22	78	56	31	147	116	13.1	42.20	11.82	20.46	"
G 626	XXV	♀	D	21	77	56	33	105	72	13.6	39.84	11.44	19.02	"
G 627	XXVI	♀	D	20	76	56	34	133	99	13.6	38.14	12.71	20.55	"
Averages							32	123	91	13.3	39.99	12.27	20.45	
G 632	XXIII	♀	E	22	78	56	32	—	—	—	—	—	—	Severe osteoporosis
G 633	XXIV	♂	E	22	78	56	33	—	—	—	—	—	—	Not examined
G 634	XXV	♂	E	21	77	56	28	106	78	12.0	38.84	11.48	18.79	Nearly normal
G 635	XXVI	♀	E	20	76	56	24	108	84	12.0	40.84	11.64	19.70	" "
Averages							26	107	81	12.0	39.84	11.56	19.24	
G 641	XXIII	♀	F	22	78	56	31	96	65	15.1	35.55	12.66	19.67	Nearly normal
G 642	XXIV	♀	F	22	78	56	33	128	95	15.1	41.62	11.25	18.69	" "
G 643	XXV	♂	F	21	77	56	38	141	103	13.3	40.63	11.26	18.97	" "
G 644	XXVI	♀	F	20	76	56	26	105	79	13.3	43.34	11.37	20.06	" "
Averages							32	117	85	14.2	40.28	11.63	19.35	

Gross Autopsy Findings.

The irradiated rats on diets *AA*, *B*, *C* and *D* were well developed and nourished and presented no gross abnormalities of their bones. The two rats on diet *E* which died early in the experiment had acute haemorrhagic enterocolitis and their bones were thin and very brittle. The remaining two rats on diet *E* and those on diet *F* differed from those on the other four diets only in

the fact that their bones were slightly thinner and not quite as firm. One rat on diet *F* was found pregnant with eight well-developed embryos. This is interesting, since it is well known that on a - *A* diet without irradiation rats rarely become pregnant.

The non-irradiated rats on diet *AA* were normal in every respect. Grossly the bones resembled those of the irradiated rats on diets *AA*, *B*, *C* and *D*. On diet *B* two of the non-irradiated rats showed very slight enlargement of the costo-chondral junctions and some indistinctness and irregularity of the costo-chondral line. The same occurred in two of the non-irradiated rats on diet *C* and in one of these fractures of several ribs were present. In all the non-irradiated rats on diet *D* the costo-chondral junctions were very slightly enlarged, the costo-chondral lines were not quite distinct, and the costal portion of the ribs was easily bent and broken, but no spontaneous fractures were present. On each of the diets *E* and *F* two rats had numerous spontaneous fractures of the ribs. The costo-chondral junctions were moderately enlarged in all, and the costo-chondral lines in most were very indistinct and irregular. The costal portion of the ribs and the shafts of the long bones were easily bent and broken.

Histology.

In a former communication one of the authors [Goldblatt, 1923] gave a full description with photographic illustrations of the various degrees of bone pathology which occurred in non-irradiated rats on diets similarly graded in their content of fat-soluble organic factor. The same descriptions and illustrations hold true for the diagnoses given in Tables II and III of this communication. But on every deficient diet in the present study the number of rats that developed a degree of rickets or of osteomalacia was greater than in the previous investigation (see Table I). This is undoubtedly attributable to the fact that the litters were very large and that, as was stated in the beginning, the mothers of these litters were kept on a diet not very rich in fat-soluble organic factor during pregnancy and lactation. Of the rats that were irradiated daily, not one developed the slightest sign of rickets or of osteomalacia. Of those that were on diets *AA*, *B*, *C* and *D*, the bones were normal in every respect and could not be distinguished. The bones of the irradiated rats on diets *E* and *F* were nearly normal, differing from the others only in the fact that the cortical bone was slightly thinner and the trabeculae shorter, thinner and fewer, though very well calcified. This tendency for the trabeculae to be thin was noticed in some of the irradiated rats on all of the deficient diets (*B*, *C*, *D*, *E*, and *F*).

Investigation of the Calcium Content of the Bones.

Chemical analysis for calcium content of both femora, tibiae and fibulae of all the rats was made by Aaron's method, as described in a previous communication by Goldblatt [1923]. The detailed results are given in Tables II and III, and the averages in Table IV. They show that in the non-irradiated

rats a gradation occurred in the percentage of calcium deposited in the bones which corresponded roughly with the gradation in the fat-soluble organic factor content of the diets. This is a similar result to that obtained previously [Goldblatt, 1923], but the gradation is not quite as regular. The percentage of calcium in the bones of the non-irradiated rats on diet *AA* was slightly less than that of the irradiated rats on the same diet, but the difference was very slight, being only 2.9 % less per wet and 3 % per dry weight of the bones. In a previous experiment the authors [Goldblatt and Soames, 1922] showed that rats of good stock, if kept on a perfectly normal diet in darkness, grow as well and their bones develop and calcify as well as those kept in a well-lighted room supplemented with irradiations by a mercury-vapour quartz lamp for ten minutes daily. The slight difference noted above is probably due to the fact that the rats used did not have a large pre-experimental store of fat-soluble organic factor. Rats of that kind were used expressly in this investigation in order to give a very severe test to the efficacy of the light rays.

In the bones of the irradiated rats on diets *AA*, *B*, *C* and *D*, little or no difference was found in the average percentage of calcium, it being normal in the four groups for rats of that age and breeding (see Table IV). The bones of the irradiated rats on diets *E* and *F* did not calcify quite as well as the others, though very much better than those of the non-irradiated rats on the corresponding diets.

Table IV.

Diet	In bones of non-irradiated rats			In bones of irradiated rats		
	Average % H ₂ O	Average % Ca wet	Average % Ca dry	Average % H ₂ O	Average % Ca wet	Average % Ca dry
<i>AA</i>	43.01	11.77	20.77	43.27	12.12	21.40
<i>B</i>	45.95	9.89	18.24	41.30	12.02	20.49
<i>C</i>	47.64	8.41	16.03	39.59	12.06	19.86
<i>D</i>	46.05	8.60	15.89	39.99	12.27	20.45
<i>E</i>	47.53	7.93	15.09	39.84	11.56	19.24
<i>F</i>	46.65	8.01	15.02	40.28	11.63	19.35

SUMMARY AND CONCLUSIONS.

The results of macroscopical, microscopical and chemical investigation agree in showing that rats on a diet as deficient in fat-soluble organic factor as even diet *D* of this study, can grow normally and their bones develop and calcify normally provided they are irradiated for about ten minutes daily by a mercury-vapour quartz lamp.

But the light rays cannot act as a substitute for the fat-soluble organic factor. When the latter is completely absent from the diet (diet *F*), irradiation, though it causes rats on that diet to grow much better and their bones to develop and calcify much better than non-irradiated controls, yet does not bring about absolute normality in either respect. The degree to which such rats approach the normal probably depends upon the pre-experimental store of fat-soluble organic factor which the animals possess.

When rats are used which have a poor pre-experimental store of fat-soluble organic factor, if they are not irradiated, grading the content of that factor in their diet results in a corresponding gradation of the percentage of calcium in their bones. Some of the non-irradiated rats on the diets deficient in the organic factor (*B*, *C*, *D*, *E* and *F*) develop slight to moderate degrees of osteomalacia or rickets, complicated in most instances by some degree of osteoporosis. This confirms the results obtained in another investigation by one of the authors [Goldblatt, 1923].

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LXXX. THE DISTRIBUTION OF CARNOSINE IN THE MUSCLES OF THE NORMAL AND DECEREBRATE CAT.

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CARNOSINE is noteworthy as being the only tissue constituent containing a β -amino acid. Its biological significance is as yet obscure. The work described in the present paper forms part of an endeavour to decide whether the base suffers a change of concentration during changes in the metabolic conditions of muscles.

An entirely satisfactory method for its estimation is yet lacking. Skworzow first attempted quantitative determination of carnosine by estimating the nitrogen content of the carnosine silver fraction from muscle extract. Mauthner [1913] unsuccessfully attempted the quantitative isolation of carnosine as the copper salt which had been prepared previously by Gulewitsch. Fürth and Hryntschak [1914] first attempted to found a colorimetric estimation of carnosine nitrate on the fact that histidine when treated with diazobenzene sulphanilic acid and sodium carbonate, gives rise to an intense red colour. But the results given by these authors show that the method was decidedly inaccurate. Koessler and Hanke [1919] described a colorimetric method for the estimation of iminazole derivatives based on the diazo reaction and claimed a fair degree of accuracy and facility for the method. The chief modification introduced by them was the substitution of the standard histidine solution by a standard consisting of a mixture of methyl-orange and Congo-red ("CR . MO"), which gave a colour matchable with diazotised histidine. Clifford [1921] suggested a further modification of Koessler and Hanke's method. The latter authors obtained a perfect linear relationship between the amounts of histidine diazotised and the reading of the standard (CR . MO) cylinder. Clifford, however, using standard solutions of histidine and carnosine matched against a CR . MO indicator solution found that linear relationship between depth of colour and amounts of carnosine diazotised did not hold.

On the other hand, Hunter [1921] a little later confirmed Koessler and Hanke's statements.

Hunter found, however, that solutions containing carnosine, after heating to 100° for one hour, yielded less colour than the original solution, and further that filtration through three filter papers in succession caused a loss of 44 %

of the carnosine present. He also declared there is reason to believe that part of the colour obtained is due to substances other than carnosine present in the muscle extract. Proteins had been found by Koessler and Hanke to lower the reading of a standard histidine solution by 30 %.

It is clear, then, that the colorimetric estimation of carnosine is not yet placed upon a satisfactory basis, but requires further study.

According to Clifford [1921] carnosine is absent from the flesh of all invertebrates, white and flat fishes, chelonians, and birds belonging to the finch and owl tribes, but is found in fishes rich in fat, and most reptiles and amphibians, in birds, and in all mammals. The amount present is practically constant in individuals of any one species.

After keeping the muscles of mammals and fishes under ordinary conditions for four days after death, Clifford found no decrease in amount of carnosine originally present, but putrid meat yielded smaller amounts.

The claim of Pekelharing and his co-workers that in the metabolism of Muscle Tonus there is an increase of creatine seemed to make it desirable to determine if other nitrogenous metabolites in the tissue undergo similar changes and the research here reported deals with the carnosine contents of the leg muscles of cats under normal condition and in decerebrate rigidity respectively. The determination was carried out chiefly by Clifford's modification of Koessler and Hanke's method. The author has, however, slightly modified this method as will be seen from the following description.

Method of Extraction employed.

5-10 g. of finely divided muscle were placed in an Erlenmeyer's flask of about 300 cc. capacity and extracted with 100 cc. of distilled water at 70° for 30 minutes, the extract being poured into a 500 cc. flask. The residue was ground to pulp and extraction as above was repeated three times. Finally the residue was transferred to the half-litre flask and the whole made up to about 450 cc. with distilled water. The extraction was allowed to continue for one hour at room temperature with frequent shaking, and the flask was then heated 30 minutes at 100°. After cooling, the contents of the flask were made up to the 500 cc. mark with water and then filtered. The first 20 cc. or so of the filtrate were rejected. Of the main bulk 50 cc. were placed in a dry flask and 10 cc. of a 20 % solution of metaphosphoric acid were added to precipitate the soluble protein which otherwise prevents the colour development of carnosine. The mixture was left to stand 2-24 hours.

The solution was then filtered from the precipitated protein through a dry filter paper into a dry beaker. About 20 cc. of the first filtrate were again rejected to avoid any lowering of the result due to absorption by the filter paper. 30 cc. (corresponding with 25 cc. of the original extract) were placed in a 50 cc. volumetric flask and neutralised to litmus paper with 10 % caustic soda solution and made up to the mark with distilled water. Of this filtrate 0.1-1.0 cc. was used for the colorimetric determinations. The amount thus

measured (x cc.) was diluted with $(1 - x)$ cc. of distilled water; $x/2$ cc. will be seen to represent the corresponding amount of the original extract.

Standardisation of Standard Indicator. A solution of carnosine nitrate containing 0.00001 g. of the salt in 1 cc. was used as a primary standard for the preparation of the Congo red-methyl orange solution which was employed as the permanent standard. It was diazotised in one of the vessels of a Duboscq's colorimeter. Placing $(1 - x)$ cc. of distilled water, 5 cc. of 1.1 % sodium carbonate solution and 2 cc. of diazotised sulphanilic acid solution, prepared according to Koessler and Hanke, in the test cylinder, x cc. of the standard carnosine nitrate solution was added from a burette. 8-10 minutes are sufficient to develop the maximum colour. This solution was then placed on one side of the colorimeter and the scale set at 10 mm. The CR. MO solution under preparation was then compared with it.

Different amounts of carnosine nitrate were treated as above and matched against the indicator solution. The results obtained were as follows:

Carnosine nitrate in test cylinders		Reading of standard indicator	Theoretical reading of standard indicator
x cc.	mg.	mm.	mm.
0.3	0.03	9.6	9.6
0.4	0.04	13.0	12.7
0.5	0.05	16.5	16.0
0.6	0.06	21.8	19.2

No sharp linear relationship between the amount of carnosine and the reading of standard indicator was therefore found but the proportionality was nearer than in Clifford's experiments. From these figures which were consistently obtained the amounts of carnosine nitrate corresponding to any depth of the standard indicator between 9.6 and 21.8 can be obtained by calculation. As the molecular formula of carnosine nitrate is $C_9H_{14}O_3N_4HNO_3$, the amounts of carnosine will be obtained by multiplying the amounts of carnosine nitrate by 0.78286.

Determination of Carnosine in certain leg muscles of the Cat.

Results obtained from ten cats by the method described are shown in the following table:

Table I.

Cat No.	Gastrocnemius	Quadriceps	Semi-membranus Carnosine %	Triceps	Solens	Tensor
1	1.293	0.955	1.799	0.845	—	1.299
2	1.153	0.889	1.441	0.766	0.352	—
3	1.028	1.006	—	0.706	—	—
4	1.012	—	—	0.727	0.341	—
5	0.924	0.843	1.532	0.686	—	—
6	0.905	0.687	1.221	0.574	0.483	—
7	0.775	—	—	0.753	—	—
8	0.747	—	1.578	0.669	—	—
9	0.697	0.550	—	0.602	—	—
10	—	—	1.086	0.525	—	—

As the above table shows, markedly different amounts of carnosine are present in different muscles. The semimembranus contains the largest amounts

and the solens the smallest. These differences are consistent, in spite of the fact that the absolute amount of the base in any one muscle is seen to differ considerably when one animal is compared with another. Clifford did not find this wide variation in different animals. In regard to this point further investigation is therefore required. In favour of the reliability of the method as used by myself (at least when comparisons are in question), I would point to the close agreement between the amounts of carnosine found in the experiments now to be described. In these the muscles of opposite sides were compared in the same animal.

As stated above, the experiments were done in an endeavour to decide whether the phenomena involved in Tonus produce any change in the concentration of nitrogenous metabolites other than creatine. The particular experiments described in the present paper deal with a comparison between normal muscles and those in the condition of decerebrate rigidity. As a considerable difference exists in the amounts of carnosine in the same muscle in different cats (Table I), it was necessary to compare the amounts of the base on opposite sides of the same animal. On one side the occurrence of rigidity was prevented by cutting the motor nerve.

As a preliminary, a comparison was made of the muscles of normal cats on opposite sides (Table II). Next, also in normal cats, the effect of cutting the motor nerves on one side was investigated, estimations being made four hours after section of the nerves (Table III). Finally, in other animals, the nerves of one side were cut and decerebration then performed. Estimations were then made after the rigidity had existed for four hours (Table IV).

It is a well-known fact that the decerebrate rigidity can be seen in gastrocnemius, quadriceps and triceps. The estimation was made with these three muscles. The next three tables show the results respectively. Decerebration was carried out by Dr K. Uemo.

Table II.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Left	1.030	1.012	0.714
	Right	1.025	1.001	0.699
	Difference	0.005	0.011	0.015
2	Left	0.684	0.546	0.596
	Right	0.697	0.550	0.603
	Difference	- 0.013	- 0.004	- 0.007
3	Left	0.769	0.696 0.533	0.753
	Right	0.775	0.690 0.525	0.740
	Difference	- 0.006	0.006 0.008	0.013

Table III.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Left (nerve cut)	—	0.736	0.666
	Right (normal)	—	0.749	0.671
	Difference	—	- 0.013	- 0.005
2	Left (nerve cut)	—	0.832	0.667
	Right (normal)	0.924	0.843	0.689
	Difference	—	- 0.011	- 0.019 (- 0.022)
3	Left (nerve cut)	—	0.683	0.579
	Right (normal)	0.905	0.687	0.574
	Difference	—	- 0.004	0.005

Table IV.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Dec. rigidity	0.850	0.554	0.517
	Nerve cut	0.843	0.555	0.520
	Difference	0.007	- 0.001	- 0.003
2	Dec. rigidity	1.101	0.797	0.677
	Nerve cut	1.105	0.795	0.669
	Difference	- 0.004	0.002	0.008
3	Dec. rigidity	1.300	0.961	0.849
	Nerve cut	1.296	0.950	0.840
	Difference	0.004	0.011	0.009

It will be seen on examination of the above figures that neither cutting the motor nerve nor the induction of decerebrate rigidity has any effect upon the carnosine in the muscles.

SUMMARY.

Using a colorimetric method based upon that of Koessler and Hanke, as modified by Clifford, estimations of carnosine have been made in the muscles of the cat.

The method gave highly consistent results when applied to corresponding muscles in the opposite limbs of the same animal.

The carnosine content of any one type of muscle varies considerably in different cats. Much wider differences, however, are to be observed in different muscles of the same animal, and a high or low concentration respectively would seem to be characteristic of individual muscles.

Neither section of its motor nerves nor the condition of decerebrate rigidity produces any effect upon the concentration of carnosine in muscle.

I wish to express my grateful thanks to Professor F. Gowland Hopkins for the interest he has taken in this work.

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LXXXI. THE PROPERTIES OF COLLOIDAL GUM BENZOIN.

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INTRODUCTION.

IN 1920 a new precipitation test for the cerebro-spinal fluid was introduced by Guillain, Laroche and Lechelle [1920]. This test is carried out in a manner similar to the Lange colloidal gold test [Lange, 1912]. A colloidal solution of Sumatra gum benzoin¹ is added to an equal volume of salt-solution containing varying amounts of cerebrospinal fluid. In certain of the tubes, flocculation and sedimentation of the 'benzoin' takes place, and from this, indications are said to be obtained of the nature of the disease. The zones of precipitation vary in position but more particularly in intensity, and are difficult to explain. It was with the hope of obtaining some knowledge of the mechanism of these precipitations that an investigation of the colloidal properties of 'benzoin' sol was undertaken.

Macheboeuf [1921] and Guillain, Laroche and Macheboeuf [1921] have determined that the charge on the particles in the sol is negative, but otherwise very little information is available on the subject.

In view of the fact that the behaviour of gelatin has been so elaborately studied and that this material could be obtained in relatively pure form by Loeb's [1922, 1] method this protein was first used in order to gain information as to the effect of proteins on 'benzoin.' The interesting fact was discovered, that in great dilution gelatin caused precipitation of the sol. This property, it has been found, is not peculiar to gelatin, and a similar precipitation occurs in presence of very dilute solutions of other proteins, *e.g.* haemoglobin, of the proper concentration. Neisser and Friedemann [1904] and Bechhold [1904] had shown that small amounts of gelatin rendered mastic precipitable by amounts of sodium chloride which, alone, were incapable of causing precipitation. Similar effects had also been noted by Walpole [1913] regarding the interaction of acid, gelatin and mastic sol or an emulsion of oil in water. Zsigmondy in 1901 discovered that proteins had a definitely protective action against the precipitation of solutions by electrolytes while, according to Zunz [1904], certain proteoses are, on the other hand, capable of causing precipitation. The 'benzoin' sol therefore showed itself to be somewhat remarkable

¹ Afterwards in this paper referred to as 'benzoin.'

in that it is precipitated by gelatin, without acid or salt, and, accordingly, this point has been more fully investigated. It is, however, to be noted that Gann [1917] was able to demonstrate precipitation by gelatin of gold sols which had an acid reaction but not of sols which were alkaline and considered that the hydrogen ion concentration was an important factor. In a recent summary of experimental results Pauli [1922] has stated that pure proteins, free from electrolytes, have no protective power but, on the other hand, cause precipitation of colloids.

From the study of the effect of proteins in precipitating the sol, we have been led to investigate its precipitation by electrolytes. It is well known that the power of electrolytes to cause precipitation of a colloid depends largely on the valency. This is well shown by results obtained by Tartar and Gailey [1922] who found that in the case of mastic sol precipitation by acid always occurred at a p_H of about 2.6, independently of the actual acid used, or the actual amount added. At any p_H greater than 2.6, no precipitation occurred. We have obtained exactly similar results with a 'benzoin' sol, so that a protective effect of the acid anion would not appear to occur. Tartar and Gailey have also shown, using the same mastic sol, that in precipitation by salts, the p_H of the salt solution is of great importance and may largely account for differences attributed to a special protective effect of the anion. With, for example, sodium acetate, the p_H is high, and high concentrations of the salt are required to precipitate, but if the p_H is brought to a constant value by adding the acid corresponding to the salt, *e.g.* acetic acid in the case of sodium acetate, the same equivalent amount of salt is effective. We have treated the question from a slightly different point of view, as we have found the amounts of a standard electrolyte, sodium chloride, required to precipitate at various hydrogen ion concentrations, and compared the amounts of other salts necessary to precipitate with the amount of sodium chloride required at the p_H produced by the new salt. Our results, it will be seen, confirm those of Tartar and Gailey.

Closely connected with the precipitating effect of gelatin on 'benzoin' is its protective power. This protective action of gelatin is of course well known and has been extensively investigated, particularly with regard to the gold sol. From our observation on the effect of gelatin on 'benzoin' it will be seen that considerably higher concentrations of gelatin are required to protect than to precipitate, confirming the statement made by Neisser and Friedemann.

The obvious suggestion is that with increasing concentrations of gelatin, increasing amounts are absorbed on the 'benzoin' particles. If the gelatin is positively charged, the negative charge on the 'benzoin' particles should decrease, until the zone of precipitation occurs. With more gelatin, the particles now coated with gelatin ought to be positively charged and they should commence to show protection. Full protection should set in when sufficient gelatin is absorbed to form a film round the particles, that is, at higher concentration than is sufficient to cause precipitation. If, however, the gelatin is negatively

charged, no such zone of precipitation ought to occur. According, however, to the work of Loeb [1922, 1] gelatin is positively charged on the acid side of the isoelectric point ($p_H = 4.7$), and negatively charged on the alkaline side of the isoelectric point. Hence, on the alkaline side of the isoelectric point, no precipitation ought to occur. It was, therefore, of the greatest interest to investigate the effect of change of the p_H on the precipitating and protecting effect of gelatin. The details will be found in the experimental part of the paper, but it may be said that, in general, the results to be expected are obtained. In particular, the zone of precipitation of 'benzoin' by gelatin suddenly disappears when the p_H exceeds 4.7. On the other hand, if oxyhaemoglobin, which behaves similarly to gelatin in precipitating 'benzoin,' is used, the zone persists until the p_H is over 6.

The experimental work which follows naturally divides itself into four parts:

1. General.
2. Precipitation of the sol by electrolytes.
3. Precipitation of the sol by gelatin.
4. Protection of the sol by gelatin.

I. GENERAL.

The 'benzoin' sol used by Gullain, Laroche, and Lechelle [1922], is prepared from an alcoholic extract of Sumatra gum benzoin, 10 g. of powdered crude Sumatra gum benzoin and 100 cc. of absolute ethyl alcohol, being allowed to stand at ordinary temperature for forty-eight hours. 0.3 Cc. of the filtered extract, which can be kept indefinitely, is added drop by drop with vigorous stirring to 20 cc. of distilled water at 35°. The white milky sol obtained is cooled to room temperature when it is ready for use. As, however, it was not homogeneous even to the naked eye but possessed a scum of fine particles, slight modifications were introduced in order to obtain a uniform sol for experimental purposes. After a few experiments it was found that the sol, if prepared at 55° instead of 35°, could be filtered readily through a folded No. 1 Whatman filter paper, whereas the sol prepared at 35° did not filter readily and yielded a very weak filtrate. Accordingly, the sol used in the following experiments was prepared by adding, at 55°, 0.3 cc. of the alcoholic extract drop by drop to 20 cc. of water, with vigorous stirring, care being taken to drop the extract directly into the liquid without touching the side of the vessel. The fluid obtained was filtered at once through a folded filter paper, and the filtrate used after cooling to room temperature. In the initial stages of the work, fresh sol was prepared for each experiment, but later it was found that this was not necessary as the properties of the sol did not change on standing for a few days.

The sol so prepared is slightly less sensitive to precipitation by sodium chloride than is the original, as shown by the following table. 1 Cc. of sol is

added to each of a series of small test-tubes, each containing 1 cc. of a solution of sodium chloride. The tubes are immediately shaken and allowed to stand overnight at room temperature. The strengths of the solutions of sodium chloride in the various tubes are given.

Table I.

Effect of mode of preparation on sensitiveness of sol to salt precipitation.

Concentration of NaCl	0.01 %	0.02 %	0.03 %	0.04 %	0.05 %	0.06 %
Sol prepared at 35°, unfiltered	0	2	4	4	4	4
" 35°, filtered	0	0	0	0	2	4
" 55°, unfiltered	0	0	2	3	4	4
" 55°, filtered	0	0	0	0	4	4

The degree of precipitation in the tubes in these and in the following experiments is denoted by numbers:

4 = complete precipitation;

3 = almost complete precipitation, a slightly opalescent fluid being left;

2 = partial precipitation, the fluid being milky;

1 = slight precipitation, some of the precipitated sol being visible at the foot of the tube, but little change being observable in the liquid left. Sometimes differences in opacity were observed, without definite flocculation or sedimentation, but usually these have been neglected.

The test-tubes were of hard glass, and before being used in any experiment were thoroughly cleaned by standing at least sixteen hours in sulphuric acid-dichromate mixture, followed by washing out each one separately about twelve times in tap-water, after which they were immersed for some time in tap-water and then in freshly distilled water. They were touched throughout only with clean nickel tongs. They were then allowed to drain in crates lined with filter paper.

Unless otherwise stated, the tubes were well shaken after the various solutions had been added, and were then allowed to stand overnight, so that they were read the following day.

The sol, as prepared for these experiments, is an apparently homogenous, milky fluid, of great stability. No apparent settling takes place even after standing for weeks. The sol is definitely acid, 0.3 cc. of *N*/50 NaOH being required to neutralise 10 cc. of it (using methyl red as indicator). When seen under the ultra-microscope, the 'benzoin' particles appear of fairly uniform size, in vigorous Brownian movement, and the observation of Macheboeuf [1921] that they are negatively charged has been verified repeatedly. A very rough count indicated that the diameters of the particles were of the order 0.3μ ; precise estimations were not attempted. They may be seen with the ordinary dark ground illumination apparatus.

The determination of hydrogen ion concentration in the following experiments was carried out colorimetrically using the Clark-Lubs sulphonephthalein series of indicators, an electrometric apparatus not being available [Clark,

1920]. This meant that determinations could be made only in the supernatant fluid, after precipitation had occurred, as it did not appear possible to make readings in the presence of the sol. It is unlikely, however, that the hydrogen ion concentration of the supernatant fluid differs from that of the mixture before precipitation, as it is shown later that in the case of precipitation by calcium chloride or by silver nitrate, the amount of calcium or silver carried down with the precipitate is at most very small. Now a change of 0.1 in the p_H means a change of about 25 % in the actual hydrogen ion concentration, and so it is very improbable that hydrogen ions are absorbed by the precipitate in quantities sufficient to cause any significant change in the p_H .

Readings were at first made with buffer mixtures, but it was found that very good readings were obtained by the use of the coloured plate given in the book by Clark [1920]. With practice good and consistent readings are obtained, and most of the readings recorded have been obtained by this method.

II. PRECIPITATION OF THE SOL BY ELECTROLYTES.

Acids. Tartar and Gailey [1922] found that the precipitation of mastic sol by acids was largely if not entirely, dependent on hydrogen ion concentration, precipitation occurring when the p_H of the solution was less than 2.6 or 2.8. The following experiment shows that 'benzoin' gives a similar result.

To 5 cc. of acids of various strengths was added 5 cc. of 'benzoin' sol. After precipitation was complete the p_H was determined in the supernatant fluid of the tube which showed complete precipitation in the presence of the smallest amount of acid.

The results obtained were as follows:

Hydrochloric acid	2.6
Acetic	2.8
Sulphuric	2.6
Oxalic	2.8
Citric	2.8

It will be seen that, within the experimental error of the methods employed, precipitation by acids depends on the hydrogen ion. The mineral acids appear to be slightly less effective than the organic acids, but it is very doubtful if the difference has any significance. In any case, it is usually the ions of the organic acids that are said to exert antagonistic and protective action [cf. Taylor, 1920], as much larger amounts of equivalent solutions of the sodium salts of these acids are required to precipitate than of sodium chloride. They certainly seem to exert no protective action against precipitation by the hydrogen ion. It would, in fact, appear that organic anions as such do not protect, but that, as Tartar and Gailey suggest, the anomalous behaviour of their salts is due to alkalinity.

To investigate this further a large number of experiments has been carried out. Determinations have been made of the minimum amounts of various salts which, in a volume of 1 cc., were required to produce precipitation of 1 cc.

of 'benzoin' sol. The maximum amount failing to produce precipitation is also recorded. In Table II are recorded the percentage strength of the solutions added and also the amount of salt in these solutions calculated in terms of gram equivalents $\times 10^6$ (b and b_1 in Table II).

Table II.

Precipitation by electrolytes.

	Maximum salt concentration not giving precipitation		Minimum salt concentration giving precipitation		p_H of supernatant fluid
	a = actual concentration of salt solution %	b g. Eq. $\times 10^6$ per cc.	a_1 = actual concentration of salt solution %	b_1 g. Eq. $\times 10^6$ per cc.	
NaCNS	0.025	3.1	0.05	6.2	4.2
NaBr	0.05	4.9	0.075	7.3	4.2
$\text{Na}_2\text{Fe}(\text{CN})_6 \cdot 2\text{H}_2\text{O}$	0.075	5.0	0.125	8.4	4.2
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	0.14	8.7	0.15	9.3	—
$\text{NaI} \cdot 2\text{H}_2\text{O}$	0.125	6.7	0.175	9.4	4.2
NaIO_3	0.15	7.6	0.2	10.1	4.2
NaCl	0.04	6.8	0.06	10.2	4.2
NaClO_3	0.075	7.0	0.125	11.7	4.2
NaNO_3	0.05	5.9	0.1	11.8	4.2
NaClO_4	0.125	10.2	0.2	11.3	4.2
$\text{Na}_4\text{Fe}(\text{CN})_6 \cdot 12\text{H}_2\text{O}$	0.15	11.5	0.225	17.3	5.4
NaF	0.05	11.9	0.875	20.8	4.5
$\text{Na}_2\text{C}_2\text{O}_4$	0.2	29.8	0.25	37.3	5.4
Na_2 tartrate $\cdot 2\text{H}_2\text{O}$	0.3	26.3	0.5	43.8	5.4
NaNO_2	0.15	21.7	0.3	43.5	5.6
Na acetate $\cdot 3\text{H}_2\text{O}$	1.0	73.5	2.0	147.1	6.8
Na_2 citrate $\cdot 5\frac{1}{2}\text{H}_2\text{O}$	1.5	126.1	2.0	168.1	7.4
NaCN		No precipitation up to 5 % NaCN			
$\text{K}_2\text{Cr}_2\text{O}_7$	0.005	0.34	0.015	1.0	3.8
$\text{K}_3\text{Fe}(\text{CN})_6$	0.0625	5.7	0.075	6.8	4.0
KIO_3	0.1	4.7	0.15	7.0	4.2
KBr	0.0875	7.3	0.1	8.4	4.2
K_2SO_4	0.05	5.7	0.075	8.6	4.2
KI	0.1	6.0	0.15	9.0	4.0
KCl	0.06	8.0	0.07	9.4	4.2
KNO_3	0.05	4.9	0.1	9.8	4.0
KClO_3	0.1	8.2	0.125	10.2	4.2
KCNS	0.075	7.7	0.125	12.9	4.2
$\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$	0.15	14.2	0.2	19.0	5.2
$\text{KF} \cdot 2\text{H}_2\text{O}$	0.14	14.8	0.18	19.1	4.3
K_2 oxalate $\cdot \text{H}_2\text{O}$	0.2	24.1	0.3	36.1	5.2
K_2 citrate $\cdot \text{H}_2\text{O}$	1.0	92.5	2.0	185.0	7.2

It will be seen that the figures obtained for salts with univalent cations were not constant. This is in agreement with the experimental results of others. Measurements were made of the p_H of the supernatant fluid in the tubes which just showed complete precipitation. It was then found that there was a marked relationship between the p_H of the solution and the amount of the salt required to produce precipitation of the solution.

This agrees well with the results of Tartar and Gailey [1922] for mastic sol. They added equivalent amounts of various salts to the sol and then determined the p_H to which the mixtures required to be brought in order to cause precipitation. It was found to be the same in all cases. In view of this it became of interest actually to determine the effect of p_H on precipitations

of 'benzoin' by sodium chloride. Solutions of alkali and acid of various strengths were prepared and 0.5 cc. of these added to each of a row of tubes. To each tube in the various rows were then added 0.5 cc. of sodium chloride solution of increasing strength and then 1 cc. of 'benzoin.' After precipitation was complete, the p_H of the supernatant fluid was measured in the tube showing complete precipitation with the smallest amount of sodium chloride in each row (see Table III). The amount of sodium chloride present was

Table III.

Effect of p_H on precipitation by NaCl.

		Concentration of NaCl											
		4%	2%	1%	0.5%	0.25%	0.125%	0.06%	0.03%	0.015%	0.007%	0.0037%	0.0019%
tubes of triangles of hydrochloric acid	N/400	—	—	—	—	4	4	4	4 (p_H 2.8)	4	4	4	4
	N/800	—	—	—	—	4	4	4	4 (p_H 3.2)	3	2	0	0
	N/1600	—	—	—	—	4	4	4 (p_H 3.4)	4	2	0	0	0
	N/3200	—	—	—	—	4	4	4 (p_H 3.6)	2	0	0	0	0
	N/12800	—	—	—	—	4	4	4 (p_H 3.8)	0	0	0	0	0
	Distilled water	—	—	—	—	4	4	4 (p_H 4.1)	0	0	0	0	0
	N/12800	—	—	—	—	4	4	4 (p_H 4.4)	0	0	0	0	0
t concentration of sodium hydroxide solutions	N/6400	4	4	4	4	4	4 (p_H 4.4)	1	0	0	0	0	0
	N/3200	4	4	4	4	4	4 (p_H 4.6)	0	0	0	0	0	0
	N/2500	4	4	4	4	4	4 (p_H 4.6)	0	0	0	0	0	0
	N/2000	4	4	4	4	4 (p_H 5)	0	0	0	0	0	0	0
	N/1600	4	4	4	4	4 (p_H 5)	0	0	0	0	0	0	0
	N/1400	4	4	4 (p_H 6.3)	2	0	0	0	0	0	0	0	0
	N/1200	4	4	4 (p_H 6.4)	0	0	0	0	0	0	0	0	0
	N/800	4	4	3 (p_H 6.8)	0	0	0	0	0	0	0	0	0
	N/600	4	4 (p_H 7.2)	0	0	0	0	0	0	0	0	0	0
	N/400	4 (p_H 7.4)	3	0	0	0	0	0	0	0	0	0	0
	N/200	4 (p_H > 9.5)	0	0	0	0	0	0	0	0	0	0	0

calculated. It was found that the amount of sodium chloride required to produce precipitation increased with the increase in p_H . It is, therefore, necessary in order to compare the efficiency of salts as precipitants to do so at the same p_H . In Fig. 1 curves have been drawn representing the amount of sodium chloride required at various p_H . With this is recorded a curve in which the amount of different sodium salts required to produce precipitation is plotted against the p_H of the supernatant fluid in the tube where complete precipitation just occurs.

It will be seen that though the correspondence of the curves is not quite exact, the p_H may at least account for most of the discrepancies between the various salts.

In the case of sodium citrate (p_H 7.4) the figure found is 192, whereas at that p_H the amount of sodium chloride required is 250. The difference is probably not more than the experimental error, which is large because of the small degree of buffering of the sodium chloride solutions. Moreover, the colorimetric estimations are only approximate.

One or two interesting points which arise from our experiments may be commented on here.

With ferric chloride difficulties in estimating the minimum amount of salt required to precipitate were encountered, because two zones of precipitation occurred. In the zone at higher concentrations (0.1 % solution) the p_{H} is 2.8, a degree of acidity which could of itself produce precipitation. In the second

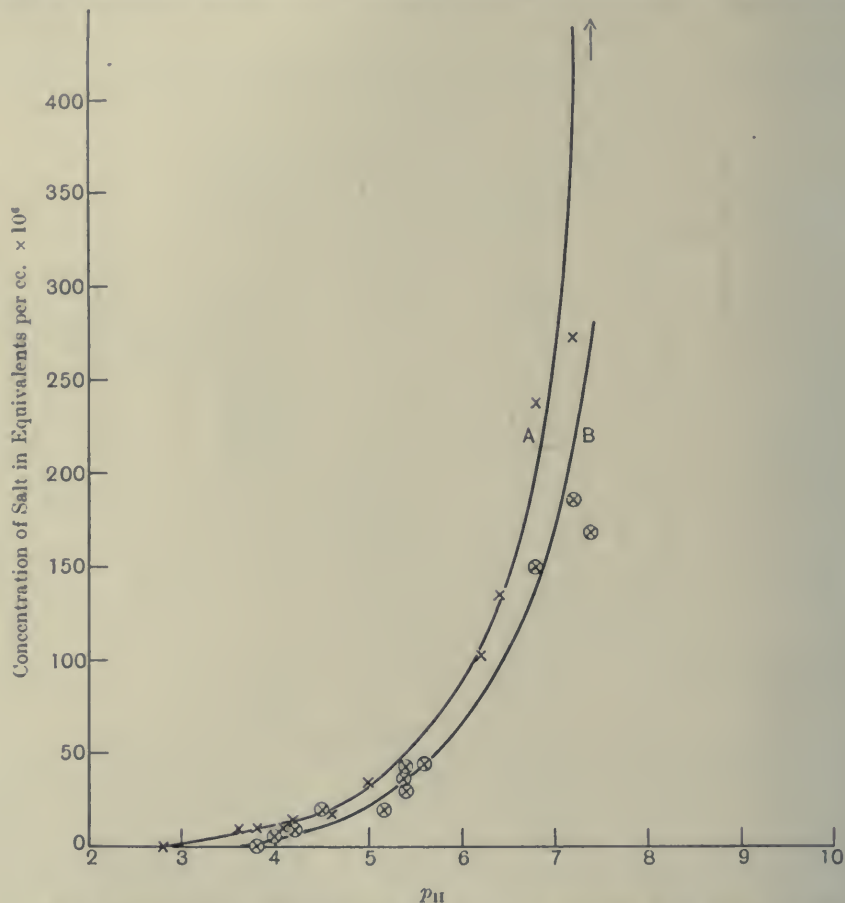


Fig. 1. Minimum concentrations of salts required to precipitate when added to equal volume of 'benzoin' sol.

x. Concentrations of sodium chloride, the p_{H} being adjusted by addition of acid or alkali.

⊗. Concentrations of various salts as detailed in Table II. p_{H} is that of supernatant fluid after precipitation of 'benzoin.'

Curve A represents concentrations of sodium chloride just sufficient to cause precipitation at various p_{H} .

Curve B is drawn from results obtained with various salts other than sodium chloride.

zone of precipitation (at 0.0015 %) the precipitation is possibly due to colloidal ferric hydroxide as in the intermediate region no precipitation occurs and the 'benzoin' particles have a positive charge. Similar results were obtained by Neisser and Friedemann [1904] with 'mastic' sol.

With aluminium sulphate, however, between concentrations of 10 % and 0.00007 % no such second zone appeared as in the case of ferric chloride.

Indeed aluminium sulphate precipitates the sol at all concentrations greater than that at which it first produces precipitation, and causes no reversal of sign of charge on the particles. Such reversal of sign in the case of ferric chloride is possibly due to adsorption of colloidal iron hydroxide on the particles. At the low concentration used, most of the ferric chloride will be hydrolysed, and so will probably be present as ferric hydroxide sol. In that case the phenomena observed will be similar in nature to the precipitation observed with gelatin, and it is being investigated at greater length.

It is to be noted that no precipitation was obtained with mercuric chloride in concentrations up to 10 %, presumably because of the low degree of ionisation of this salt. Similar results have been previously obtained using other sols, and this very strongly indicates the ionic nature of the precipitation.

With sodium cyanide and potassium cyanide no precipitation was obtained. This is no doubt due to the strong alkalinity of the solutions.

Experiments were carried out with silver nitrate and also calcium chloride solutions as precipitants of 'benzoin' in order to determine any change in the salt concentration in the supernatant fluid after precipitation of the sol.

Calcium chloride solution (approx. 0.06 %) was added to an equal quantity of the sol. After standing overnight precipitation of the sol was complete. The clear supernatant fluid was pipetted off. The amount of calcium present was estimated by the usual method—precipitation as oxalate and titration of the oxalic acid with potassium permanganate solution. In 150 cc. of the supernatant fluid was found 0.0131 g. of calcium. An estimation of the calcium present in 150 cc. original solution gave 0.0259 g. Ca, or, diluted one in two, 0.0130 g. So that, within the limits of experimental error the determinations were identical.

Similar results were obtained when silver nitrate was used as the precipitating salt, the silver in this case being estimated volumetrically.

The amount of the precipitating ion removed during the process of precipitation was apparently too small to be detected by the methods. As noted above (p. 639), these results give a basis for the practice of measuring the p_{H} of the supernatant fluid as the p_{H} at which precipitation actually takes place.

Effect of Concentration of Sol on Precipitation by Electrolytes.

Burton and Bishop [1920] and Burton and McInnes [1921] have formulated the following laws:

- (1) In the case of univalent ions, the amount required to cause precipitation increases as the solution is diluted.
- (2) In the case of bivalent ions, no such increase is observed.
- (3) In the case of trivalent ions, the amount required is approximately proportional to the concentration of the solution.

Weiser and Nicholas [1921] have, however, cast doubt on the general validity of these laws, a doubt which is apparently shared by Krnyt and van der Spek [1919]. We have determined the minimum concentrations of sodium

chloride, copper sulphate and aluminium sulphate solutions required to produce precipitation when added to 'benzoin' sols of the following strengths:

(1) 'Benzoin' of double¹ the usual strength (*i.e.* 3 cc. of alcoholic extract added to 100 cc. of water).

(2) 'Benzoin' of double the usual strength, diluted 1 in 2 with distilled water.

(3) 'Benzoin' of double the usual strength, diluted 1 in 20 with distilled water.

The results are given in Table IV, and the amount of sodium chloride required in the case of 'benzoin' of the usual strength is given for comparison.

Table IV.

Effect of concentration of sol on precipitation by kations of different valency.

Nature of sol	Concentration of solution which produces precipitation when added to an equal volume of sol		
	NaCl %	CuSO ₄ %	Al ₂ (SO ₄) ₃ %
'Benzoin,' ordinary strength	0.05-0.06	—	—
" double "	0.04-0.05	0.03-0.05	0.007-0.015
" " " diluted 1/2	0.05-0.08	0.04-0.06	0.007-0.015
" " " " 1/20	0.1-0.2	0.08-0.1	0.015-0.03

It would appear that these laws are not here applicable.

In all cases, an increased concentration of salt is required to precipitate diluted solutions.

Dilute sols are also apparently slightly less sensitive to precipitation by hydrogen ions. Direct experiment showed a slight but constant difference between the p_{H} required to precipitate a concentrated solution and that effective for a dilute one. Whereas a double strength 'benzoin' sol is precipitated when a p_{H} of 2.8 is reached, the same sol diluted 1 in 20 requires a p_{H} of 2.6 to precipitate it. This shows that the greater sensitiveness of the stronger solution to salts is not due altogether to its higher p_{H} , although there is actually a slight difference in the p_{H} of the concentrated and dilute solutions. The following experiment in which the salt content is kept constant and the p_{H} varies, also shows that this difference in p_{H} is not sufficient to account for the difference in sensitiveness to electrolytes. 1 Ce. of a dilute sol (double strength diluted 1 in 10) was added to each of ten tubes, each containing 1 cc. of sodium chloride of the concentration (0.06 %) required to produce precipitation in an ordinary sol at a p_{H} of 4.2. The sodium chloride solution contained acetic acid of strengths 1 %, 0.5 %, 0.25 %, etc., in the various tubes so as to vary the p_{H} . Precipitation appeared in those tubes having a p_{H} of 3.6 or less and not in the others. The dilute sol, therefore, appears definitely less sensitive to electrolyte precipitation than the normal one, which is precipitated by an equal volume of 0.06 % sodium chloride at a p_{H} 4.2.

¹ This 'benzoin' sol double the usual strength was used so that, when diluted 1 in 20, the resulting liquid was still opaque enough to show precipitation readily when that had occurred.

III. PRECIPITATION OF 'BENZOIN' BY GELATIN AND OXYHAEMOGLOBIN.

As mentioned in the introduction it has been found that gelatin at certain dilutions produces precipitation of 'benzoin' sols. A 1 % solution of iso-electric gelatin was prepared by the method described by Loeb [1922, 1]. On ignition this was found to leave an ash weighing 0.08 % of the dry gelatin. A series of dilutions of gelatin in distilled water was prepared varying from 1/10,000, 1/20,000 to 1/5,120,000. Each tube contained 1 cc. of gelatin solution and to this was added 1 cc. of 'benzoin' sol. Precipitation occurred in the tubes containing gelatin solution 1/320,000 and 1/640,000 and in the tubes containing gelatin solutions above and below these concentrations no precipitation occurred.

A series of experiments was carried out to study the effect of variation of p_{H} on the zone of precipitation. For the purpose a series of dilutions of gelatin, hydrochloric acid and sodium hydroxide were prepared. To each of a series of tubes were added 0.5 cc. of gelatin solution of various strengths, 0.5 cc. of the appropriate acid or alkali solution and finally 1 cc. of 'benzoin.' Readings were made when precipitation was complete and the p_{H} was estimated in the supernatant fluid. The figures in Table V represent the concentrations of acid or alkali and of gelatin in the mixture before addition of the 'benzoin.' The concentrations originally prepared were double that recorded.

Table V.

Precipitation of 'benzoin' by gelatin.

Concentration of hydrochloric acid	Concentration of sodium hydroxide	Concentration of gelatin												No gelatin
		1/2500	1/5000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/320,000	1/640,000	1/1,280,000	1/2,560,000	1/5,120,000	
N 800		0	0	0	0	0	0	0	—	—	—	—	—	
N 1600		0	0	0	0 (-)	0 (-)	0	0	—	— (-)	—	—	—	
N 3200		0	0 (+)	0	0 (+)	1 (7 +)	4	4 (p_{H} 4.7)	4	1	0	0	0	
N 12800		0	0 (+)	0	0 (+)	0 (+)	0 (+)	1	4 (p_{H} 4.1)	4 (p_{H} 4.1)	0	0 (-)	0	
N 51200		0	0	0	0	0	0	0	3	4 (p_{H} 4.1)	0	0	0	
H ₂ O		0	0	0	0 (+ +)	0	0	0 (+)	2	4 (p_{H} 4.1)	0	0	0 (-)	
N 51200		0	0	0	0	0	0	0	1	4 (p_{H} 4.1)	1	0	0	
N 12800		—	—	—	—	0	0	0	1	4 (p_{H} 3.8)	1	0	0	
N 3200		—	—	—	0 (+ + +)	0	0 (+ +)	0	1	4	4 (p_{H} 3.6)	2	0	
N 1600		—	—	—	—	—	—	0	1	4	4 (p_{H} 3.5)	3	0	
N 800		—	—	—	—	—	—	0	1	4	4 (p_{H} 3.2)	4	3	
N 400		—	—	—	—	—	—	0	1	4	4 (p_{H} 2.8)	4	4	
N 200		—	—	—	—	—	0	0	0	1	—	—	—	
N 100		—	—	—	—	—	0	0	1	2	—	—	—	
N 40		—	—	—	—	—	0	1	3	4	—	—	—	
N 10		—	—	—	0	0	1	4	4	4	—	—	—	

(+) and (-) indicate sign of charge on the particles.

It may be noted that in these experiments as in those on precipitation by electrolytes, the p_{H} recorded is that of the supernatant fluid. The 'benzoin' solution is itself acid, and so the p_{H} of the gelatin solution before the 'benzoin' is added is of no value in giving the p_{H} at which the precipitation actually occurs. The p_{H} of the supernatant fluid suggests itself as the best measurement of the p_{H} at which precipitation occurs.

Fig. 2 shows the p_H plotted against concentration of gelatin. It will be seen that as the acidity is increased a decrease occurs in the concentration of gelatin at which precipitation takes place until a p_H of 2.8 is reached when the zone

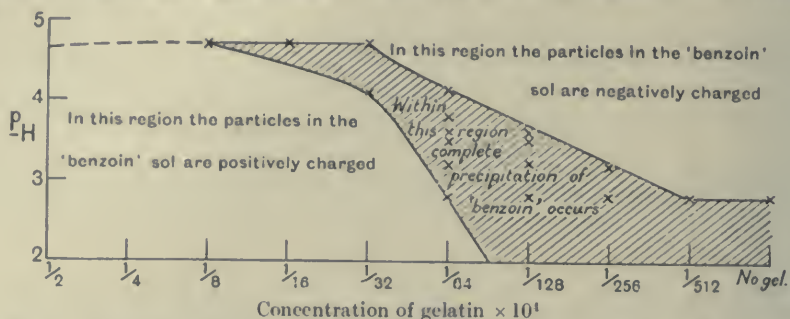


Fig. 2. Illustrating effect of p_H on precipitation of 'benzoin' by gelatin. (N.B. The concentration of gelatin given is that of solutions before addition of equal volume of 'benzoin' sol.)

effect disappears, the acid itself precipitating the 'benzoin' solution, the gelatin now appearing in the rôle of a protective colloid. On increasing the p_H on the other hand the precipitating zone is found in higher concentrations of gelatin. As the isoelectric point of gelatin is reached (p_H 4.7) the zone broadens, but when this is passed no further precipitation of the 'benzoin' is observed.

Precipitation of 'Benzoin' by Oxyhaemoglobin.

In view of the interest attached to the precipitation of 'benzoin' by gelatin, more particularly with regard to the importance of the isoelectric point, and its predominance in the effect, it appeared to be very desirable to investigate in a similar way, a protein having a markedly different isoelectric point. Serum globulin and serum albumin were both investigated; precipitation took place at a p_H up to the isoelectric point, and beyond this, there was no precipitation. This, however, will be discussed at greater length in the succeeding paper. Oxyhaemoglobin is of greater interest as its isoelectric point differs from that of gelatin and is given as 6.6, and, therefore, these zones of precipitation should not exist at p_H exceeding 6.6, but should exist up to about that point.

A solution of oxyhaemoglobin was prepared by washing well with 0.8 % sodium chloride solution, red blood cells obtained from citrated sheep's blood, so that the washings finally gave no test for protein. The cells, which weighed moist 8.01 g., were added to distilled water, and the resulting oxyhaemoglobin solution dialysed for over thirty-six hours in a collodion sac against running tap-water. The final volume of oxyhaemoglobin solution was 85 cc. This is of course not quite pure oxyhaemoglobin solution but, particularly in high dilutions, the amount of impurity present is probably very small.

An experiment exactly similar to the experiment with gelatin described on p. 645 was carried out with this solution. The results are given in Table VI. It may be remarked that variations in different experiments were

occasionally noticed in the tubes in the first four rows. This is probably to be attributed to the great sensitiveness at this point to the slight change in reaction.

Table VI.

Precipitation by Oxyhaemoglobin.

Concentration of oxyhaemoglobin	1/6.25	1/12.5	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400
N/200	—	—	—	—	—	0	0	0	0	0	0	0	0	0	0
N/100	—	—	—	—	—	0	0	0	0	0	0	0	0	0	0
N/800	4	2	0	4 (p_H 6.3)	0	0	0	0	0	0	0	0	0	0	0
N/1000	4	4	2	4 (p_H 6.4)	2	0	0	4 (p_H 6.1)	0	0	0	0	—	—	—
N/1200	4	4	4	4	4 (p_H 6.2)	2	2	4 (p_H 6.4)	0	0	0	0	—	—	—
N/1400	4	4	4	4	0	0	0	4 (p_H 5.7)	4 (p_H 5.6)	0	0	0	—	—	—
N/1600	4	4	4 (p_H 5.8)	0	0	0 (+)	0	4 (p_H 5.8)	0	0	0	0	0	—	—
N/3200	—	—	—	—	—	0	0	0	0	4 (p_H 4.8)	4	0	0	—	—
N/6400	4	1	0	0	0	0	0	0	0	0	4 (p_H 4.8)	2	—	—	—
N/12800	—	—	—	—	—	—	—	0	0	0	4 (p_H 4.4)	0	—	—	—
Distilled water	—	—	—	—	—	—	—	0 (+)	0	0	4 (p_H 4.2)	4	0	0	0
N/3200	—	—	—	—	—	—	—	0	0	0	0	4 (p_H 3.8)	4	0	0
N/1600	—	—	—	—	—	—	—	—	0	0	0	4 (p_H 3.4)	4	0	0
N/800	—	—	—	—	—	—	—	—	—	—	0	4 (p_H 3)	4	4	3
N/400	—	—	—	—	—	—	—	—	—	—	0	2	4 (p_H 2.8)	4	4

Fig. 3 shows p_H plotted against concentration of oxyhaemoglobin.

It will be seen that the zone of precipitation which at a p_H of 3.8 appears in the tube containing the oxyhaemoglobin solution diluted 1/12,800 gradually moves to the left with increasing p_H until the p_H rises to about 6–6.4, when

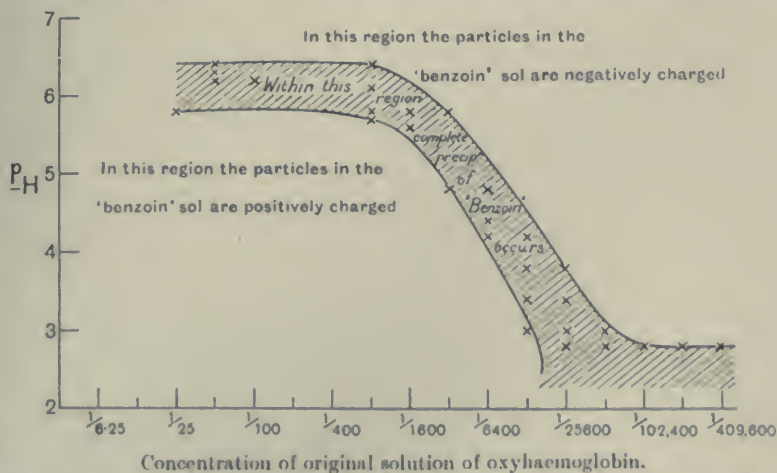


Fig. 3. Illustrating effect of p_H on precipitation of 'benzoin' by oxyhaemoglobin. (N.B. The concentration of oxyhaemoglobin is given in terms of an original solution prepared as described in the text, and is the concentration before addition of an equal volume of 'benzoin' sol.)

precipitation occurs at 1/800. A zone of precipitation at this p_H occurs with all concentrations of haemoglobin solution down to 1/6.25. The absence of any precipitation beyond the isoelectric point and presence of it up to the isoelectric point is very marked.

The effect of alteration in the reaction of the gelatin or oxyhaemoglobin in precipitation of 'benzoin' is a peculiar one. There is a general change in the position of the zone of precipitation with change of p_{H} . This is readily understood if it be assumed that the primary effect of change of the p_{H} is on the protein. In accordance with the work of Loeb gelatin exists on the acid side of the isoelectric point as a positively charged kation, on the alkaline side as an anion. If the effective precipitating agent is the positively charged gelatin ion it can be understood that little or no precipitation will occur on the alkaline side of the isoelectric point. The alteration in the position of the zone of precipitation is probably due to the fact that an optimum concentration of the gelatin is required for precipitation. This amount will be found at lower concentrations as the p_{H} decreases, as a greater part of the gelatin exists with a positive charge at the higher acidity.

That there may be some effect on the sol must be granted as acid in excess causes its precipitation, while excess of alkali dissolves it. But that this effect is not the primary one is shown by an experiment in which varying amounts of sodium chloride solution are added to the gelatin, instead of acid.

Table VII shows the result of such an experiment.

Table VII.

Influence of sodium chloride on the precipitation of 'Benzoin' by Gelatin.

The concentrations given are those of the sodium chloride and gelatin after mixing and before adding the 'benzoin.'

		Concentration of gelatin												
Concentration of sodium chloride	<div><div><div>1.0 %</div><div>0.5 %</div><div>0.1 %</div><div>0.04 %</div><div>0.02 %</div><div>0.005 %</div><div>0</div></div></div>	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	1/128,000	1/256,000	1/512,000	1/1,024,000	1/2,048,000	1/4,096,000	Water
		0	0	2	3	4	4	—	—	—	—	—	—	—
		0	0	1	2	4	4	—	—	—	—	—	—	—
		0	0	0	1	4	4	4	—	—	4	4	4	4
		0	0	0	0	1	4	4	4	4	4	3	0	0
		0	0	0	0	0	4	4	4	4	3	0	0	0
		0	0	0	0	0	0	3	4	4	3	0	0	0
		0	0	0	0	0	0	0	4	4	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0	0

It will be seen that the effect of sodium chloride on the zone of precipitation of 'benzoin' by gelatin is merely to widen it. Sodium chloride at such concentrations presumably has little effect on gelatin. It has at least no such powerful influence in altering its properties as has acid or alkali. On the other hand, the effects of sodium chloride and of hydrochloric acid on the 'benzoin' solution in the absence of gelatin are qualitatively the same, they either precipitate it or increase its sensitiveness. Hence there seems to be little doubt that the action of the acid in the above experiment must be primarily on the gelatin, because, if it were on the 'benzoin,' we should expect an effect, similar to that of the salt. This of course is also borne out by the determining influence of the isoelectric point of the protein.

Effect of Concentration of the Sol on Precipitation by Gelatin.

Neisser and Friedemann [1904] and Biltz [1904] showed that a quantitative relationship held in the mutual precipitation of oppositely charged colloids, and that this is related to a neutralisation of the charge on the particles or to a reduction of the potential differences between the disperse phase and the continuous phase. If the precipitation of 'benzoin' (negatively charged) by gelatin (positively charged) is of this nature, change in the concentration of 'benzoin' ought to cause a similar change in the concentration of gelatin at which precipitation occurs. That this actually occurs is shown by an experiment, the results of which are given in Table VIII.

Table VIII.

Effect of concentration of sol on precipitation by Gelatin.

1 cc. of the gelatin solution was added to 1 cc. of 'benzoin' solution, the mixture shaken and allowed to stand overnight.

Concentration of gelatin solution $\times 10^4$	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
'Benzoin,' ordinary strength	0	0	0	0	4 (p_{II} 4.2)	4	0	—	—	—
" double "	0	0	0	4	4 (p_{II} 4.2)	2	0	0	—	—
" " " diluted 1/2	0	0	0	0	4 (p_{II} 4.2)	4	0	0	—	—
" " " " 1/4	0	0	0	0	4	4 (p_{II} 4.4)	0	0	0	—
" " " " 1/20	0	0	0	0	0	2	4 (p_{II} 4.6)	0	0	0

It will be seen that a sol in a dilution of 1/20 of double strength ($= 1/10$ dilution of ordinary strength) requires gelatin 1/1,280,000 to produce maximum precipitation (p_{II} 4.6). Referring to Table V it will be seen that the maximum precipitation of a sol of ordinary strength with this p_{II} occurs in a concentration of gelatin of 1/160,000. Approximately, therefore, diluting the solution ten times reduces the optimum amount of gelatin to one-eighth of the amount required for the more concentrated solution, or, within the error of the experiment, the same actual amount of gelatin is required to precipitate the same actual amount of 'benzoin' at different dilutions.

Charge on the 'Benzoin' Particles.

For the purpose of observing the nature of the charge on the particles a Siedentopf-Zsigmondy slit ultramicroscope was employed. The ordinary cell was used and two fine platinum wires were introduced into this, one from each end in such a way that the end of each wire was about 2 inches from the part of the fluid observed. It was found that, to produce a definite movement, the 220-volt current was convenient, a switch, change over key and lamp being included in the circuit. The circuit was closed and the movements of the particles in the field observed immediately. The current was then reversed and a second observation made. It is essential that observations should be made immediately after closing the circuit so that interference by electrolyte disturbances may be avoided. It was found that this method was particularly useful where, as in the case of experiments with cerebrospinal fluid, only very small quantities (about 1 cc.) of liquid were available. In the crude form described, quantitative estimations were, of course, not possible but for quali-

tative determinations the arrangement was quite satisfactory. The results of such observations are indicated in Tables V and VI.

On the alkaline side of the isoelectric point of gelatin (p_H 4.7) the particles were invariably negatively charged in all concentrations of gelatin observed.

On the acid side of the isoelectric point in those concentrations of gelatin which are too great to cause precipitation, where in fact the 'benzoin' is protected, the charge on the particles is positive. The boundary between positively and negatively charged particles is within the zone of precipitation. For example, in Table V, where gelatin is diluted with distilled water, in dilutions of gelatin up to 1/1,280,000 the particles are negatively charged. At the gelatin dilution of 1/160,000 they have a slight positive charge which becomes more marked as the concentration of gelatin increases. Between these concentrations the zone of precipitation occurs.

An experiment was carried out to determine the effect of tannin in precipitating the mixtures of gelatin and 'benzoin' in the following way. Gelatin solution (1 cc. of 1/40,000 dilution) was added to 1 cc. of various strengths of sodium hydroxide solution. To each tube was added 2 cc. of 'benzoin.' This was allowed to stand overnight and precipitation observed and observation of the charges on the particles were made. The results are given in Table IX.

Table IX.

Effect of Tannin on Benzoin and Gelatin mixtures.

Sodium hydroxide	N/200	N/400	N/800	N/1600	N/3200	N/6400	N/12800	N/25600	N/51200	N/102400
Row I	0	0	0	0 (-)	2 (+?)	0 (+)	0	0	0	0

1 cc. of gelatin and 1 cc. of sodium hydroxide solution mixed, and 2 cc. of 'benzoin' added to the mixture. Read as in Row I.

From each tube 1 cc. was taken and an equal volume of a 0.2 % solution of tannin added. The tubes were read after standing overnight as follows in Row II.

Row II	0	0	4 (p_H 4.4)	4	4	4	4	4	4	4
--------	---	---	----------------	---	---	---	---	---	---	---

The acidity of the tannin solution has slightly changed the p_H and therefore the electrical charges in the mixtures, but the negative colloid (tannin) is able to precipitate the mixture only at p_H 4.4 and below, under which conditions the particles are positively charged. The 'benzoin' alone is not precipitated by the tannin under the conditions of the experiment, and no visible precipitation is given by gelatin at the concentration employed.

From these experiments it would appear that the 'benzoin' particles combine with the gelatin with the result that their electrical condition is modified. The degree of the modification is dependent on the condition of the gelatin, which again depends on the reaction. Where the charge is neutralised or reduced precipitation occurs. Where, however, the negative charge is reinforced by the gelatin or changed to a positive charge of sufficient magnitude, the sol is protected against precipitation by electrolytes. These results are in substantial agreement with those obtained by Eggerth and Bellows [1922] on bacterial and other suspensions and by Northrop and de Kruif [1922, 1, 2] on bacterial suspensions.

IV. PROTECTION OF 'BENZOIN' BY GELATIN AGAINST
 PRECIPITATION BY ELECTROLYTES.

According to the views of Bechhold [1904], and Neisser and Friedemann [1903] the protection of a lyophile colloid by a lyophile depends probably on the formation of a covering of the former round particles of the latter. The result is that the behaviour of the lyophile particles is governed rather by the properties of the protective colloid than by its own. This accounts for the fact, rather striking at first, that colloidal 'benzoin' requires little more gelatin to protect it against a large concentration of salt than is required to protect it against a small quantity. This is shown in the following experiment. Varying solutions of gelatin (1 cc.) 1/100,000, 2/100,000, etc. to 1/10,000 were introduced into a series of test-tubes. To each test-tube was added 0.2 % sodium chloride (1 cc.) and the 'benzoin' (2 cc.). A similar experiment was carried out at the same time with 2 % sodium chloride, and the results are given in Table X.

Table X.

Protection by Gelatin against precipitation by NaCl.

Concentration of gelatin \times 100,000	1	2	3	4	5	6	7	8	9	10
Concentration of sodium chloride { 0.2 %	4	4	4	0	0	0	0	0	0	0
2.0 %	4	4	4	4	0	0	0	0	0	0

It may be noted here that if any such mechanism really does occur, the 'benzoin' particles are completely protected within a few minutes. This is proved by experiments in which to each of a series of ten test-tubes was added 0.5, 1.0, 1.5, 2, etc., up to 5 cc. of gelatin (1/10,000) and distilled water to bring the total volume in each tube to 5 cc. This was followed by the addition to each tube of 5 cc. of 'benzoin' sol, of twice the usual strength (0.6 cc. of alcoholic solution to 20 cc. of water). Six rows, each containing ten tubes, were arranged so that, corresponding to each mixture of gelatin and 'benzoin,' there were six test-tubes, and into each of these six test-tubes was put 1 cc. of the corresponding mixture of 'benzoin' and gelatin, each of the six rows at this stage being of course identical. Ten minutes after the mixture of the 'benzoin' and gelatin, 1 cc. of 2 % sodium chloride was added to each test-tube in the first row and 1 cc. of 0.2 % sodium chloride solution was added to each test-tube in the second row. Thirty minutes after the mixing of 'benzoin' and gelatin, addition was made to the third and fourth row of 1 cc. of salt solution of 2 % and 0.2 % respectively, and after 2½ hours, similar addition to the tubes in the fifth and sixth rows respectively. The results are represented in Table XI, and it will be seen that protection of the 'benzoin' by the gelatin is apparently complete after 10 minutes. It is probable, however, that the protection is not instantaneous, as is shown by the fact that if the salt and gelatin be first mixed and the 'benzoin' then added, slightly different results are obtained, as is also shown in Table XI.

Table XI.

Rate of protection by Gelatin.

Concentration of gelatin $\times 100,000$	1	2	3	4	5	6	7	8	9	10
2 % NaCl added after 10 mins.	4	4	4	4	0	0	0	0	0	0
0.2 % " " 10 "	4	4	0	0	0	0	0	0	0	0
2 % " " 30 "	4	4	4	4	0	0	0	0	0	0
0.2 % " " 30 "	4	4	0	0	0	0	0	0	0	0
2 % " " 2½ hours	4	4	4	4	0	0	0	0	0	0
0.2 % " " 2½ "	4	4	0	0	0	0	0	0	0	0
'Benzoin' added to mixture of NaCl (0.2 %) and gelatin	4	4	2	0	0	0	0	0	0	0

As is to be expected, there is less protection when the 'benzoin' is added to the mixture of salt and gelatin, as in this case the salt is active as quickly as the gelatin, and effects precipitation before the protective power of the gelatin can fully exert itself.

Experiments were carried out on the amount of gelatin required to protect 'benzoin' of various strengths. A series of five rows each containing ten tubes is arranged. To each tube in each of the first four rows is added 1 cc. of a solution of gelatin, to the first, 1/100,000, to the second, 2/100,000, etc. To the test-tubes of the last row is added 1 cc. of gelatin of strength 1/20,000, 1/40,000, 1/80,000, etc., respectively. Then 1 cc. of 'benzoin' of a certain concentration is added to each tube. Single strength 'benzoin' is added to the tubes of the first row, double strength to the tubes of the second row, double strength diluted 1 in 2, 1 in 4, 1 in 20, to the tubes of the third, fourth and fifth rows respectively. All the tubes are shaken and after 15 minutes 2 cc. of 2 % sodium chloride solution is added to each tube. The results obtained after standing overnight are shown in Table XII.

Table XII.

Effect of concentration of sol on protection by Gelatin.

The concentrations of gelatin given in this table are the concentrations *before* mixing with the 'benzoin.' Sodium chloride (2 %) solution is added to the mixture of 'benzoin' and gelatin after 15 minutes.

Concentration of gelatin $\times 100,000$	1	2	3	4	5	6	7	8	9	10
'Benzoin,' ordinary strength	4	4	2	0	0	0	0	0	0	0
" double "	4	4	4	4	4	3	2	0	0	0
" " " diluted 1/2	4	4	4	0	0	0	0	0	0	0
" " " " 1/4	4	0	0	0	0	0	0	0	0	0
Concentration of gelatin $\times 10,000$	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
'Benzoin,' double strength, diluted 1/20	0	0	0	0	0	4	4	4	4	4

It will be noted in the first place that there is not much difference between the single strength 'benzoin' and the double strength diluted 1 in 2. This, of course, is to be expected. On the other hand, the various dilutions of the double strength showed marked differences in the amount of gelatin required to protect them. The double strength 'benzoin' is almost completely protected by 8/100,000 gelatin, giving a final concentration of gelatin in the experiment of $\frac{1}{4}$ (8/100,000) = 1/50,000—the factor $\frac{1}{4}$ being required, since 1 cc. of gelatin is diluted to 4 cc. by the addition of 'benzoin' and of salt, whereas

if the 'benzoin' be diluted 20 times protection occurs at a final concentration of about $\frac{1}{4}$ ($1/320,000$) = $1/1,280,000$, or about $1/25$ of the concentration required with 'benzoin' of 20 times the strength of that here used. In fact, the amount of gelatin required to protect is approximately proportional to the concentration of the sol. In interpreting the result it must be remembered that less salt is required to precipitate a concentrated sol than a diluted one, but, on the other hand, if excess of salt be used, as in this experiment, the amount of gelatin required to protect increases very slowly with increase in the actual concentration of the salt (see p. 651). It may be noted, however, that this effect may account for the small deficiency ($1/25$ instead of $1/20$) of gelatin that is required for the precipitation of the dilute sol compared with the concentrated one.

In view of the very great importance of the hydrogen ion concentration on the precipitation effect of gelatin on 'benzoin' it was of interest to investigate its effect on the protective power of gelatin. Solutions of hydrochloric acid, $N/100$, $N/200$, $N/400$... $N/16,000$, $N/64,000$, sodium hydroxide, $N/200$, $N/400$, $N/800$, $N/1400$, $N/3200$, and solutions of gelatin $1/500$, $1/1000$, $1/1600$, $1/3200$ were prepared. 0.25 Cc. of acid (or alkali), and the same amount of gelatin, were added to each one of a row of eight tubes, there being eleven rows, one for each strength of acid or alkali and one for water. 0.5 Cc. double strength 'benzoin' was then added to each tube, and readings made of those (marked * on the table) which showed incipient precipitation as indicated by increased opacity. After standing 10 minutes, 1 cc. of sodium chloride solution (2 %) was added to each tube and the tubes were allowed to stand overnight. The results obtained are shown in Table XIII.

Table XIII.

Effect of p_{H} on protection by Gelatin.

The concentrations given are the final concentrations after mixing 'benzoin,' gelatin and salt solutions.

Final concentration of gelatin $\times 1000$	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Final con- centration of sodium hydroxide								
$N/1600$	0	0	0	0	0	4	4 (p_{H} 6.4)	4
$N/3200$	0	0	0	0	0	4	4 (p_{H} 5.2)	4
$N/6400$	0	0	0	0	1	4*	4* (p_{H} 4.8)	4*
$N/12800$	0	0	0	0	4	4	4* (p_{H} 4.6)	4*
$N/25600$	0	0	0	0	2	4	4 (p_{H} 4.4)	4*
Distilled water	0	0	0	0	2	4	4 (p_{H} 4.3)	4*
Final con- centration of hydrochloric acid								
$N/51200$	0	0	0	0	1	4 (p_{H} 4.2)	4	4
$N/12800$	0	0	0	0	0	4 (p_{H} 4.1)	4	4
$N/3200$	0	0	0	0	0	4 (p_{H} 3.6)	4	4
$N/1600$	0	0	0	0	0	4 (p_{H} 3.3)	4	4
$N/800$	0	0	0	0	0	4 (p_{H} 3)	4	4*

The most interesting result of this experiment is that protection by gelatin is minimum at the isoelectric point. From the point of view that the particles of a protected colloid have many of the properties of the protective substance, this is easily understood. It is interesting to note, however, that Loeb [1923] has obtained similar results in the case of collodion particles coated with gelatin. These show a minimum of stability at the isoelectric point

of gelatin. According to Loeb this is due to the fact that gelatin is least soluble at the isoelectric point, and a similar explanation fits well the results obtained in the above experiment.

One further point is of interest. As shown above (p. 651), rather more gelatin is required to protect against precipitation by 2 % salt solution than against 0.2 % salt solution. The lower part of the table in the above experiment may be looked on as a series of experiments in protection against salt along with a definite amount of acid. It will be seen that up to a point, addition of acid does not increase the amount of gelatin required to protect. In fact, it diminishes it. The only interpretation of this result is that the acid renders the gelatin a more efficient protector, and this may be due to the gelatin anion being a more efficient protector than the uncharged neutral particle. In this case, it is not unreasonable to explain the effect of alkali on the protective power of gelatin as due to a similar increased efficiency of sodium gelatinate compared to unionised gelatin. Of course this comes well into line with the point of view of Loeb, the minimum protection at the isoelectric point being due to the small affinity for water of the isoelectric gelatin particle and the resulting small solubility. At the same time it must be remembered that alkali has itself a protective effect against precipitation by salt (see p. 641), whereas acid tends to precipitate. This may well account for the greater efficiency of gelatin as a protector in presence of alkali compared to acid, as is shown by the fact that, at a p_H of 5.2, 1/64,000 gelatin affords complete protection whereas at a p_H of 4.2 it does not, although the isoelectric point, $p_H = 4.7$, is nearer 5.2 than is 4.2.

DISCUSSION.

Colloidal solutions have for convenience been classified into two types—lyophobic colloids, such as gold sol or 'benzoin' sol which are precipitated by small quantities of salt, and are suggestive of very fine suspensions, and lyophilic colloids, on the other hand, such as gelatin solution or a solution of silicic acid which are precipitated only by large quantities of salt, and then redissolved again if the excess of salt is removed. The work of Loeb [1922, 2] has in fact shown that gelatin in certain respects may be regarded as forming a true solution, and that it behaves chemically as an amphoteric substance, combining with acid on one side of the isoelectric point ($p_H = 4.7$) and with bases on the other. The large size of the gelatin aggregate, however, prohibits it from passing through a collodion membrane, and this along with the strong attraction of the gelatin molecules for each other, aiding the formation of micelles, and tending to cause gelatin to swell rather than to dissolve, results in producing the colloidal properties of gelatin.

The 'benzoin' particles, on the other hand, have a negative charge, presumably due to the presence of some organic acid (benzoic acid) on the surface of the resin particles. These acid molecules will naturally ionise to some extent, thus producing a negatively charged particle and a slightly acid solution. The

stability of these particles is largely dependent on the electric charge. Loeb [1922, 2] who uses collodion particles shows that a dispersion of these becomes unstable if the potential difference between particle and liquid is less than about 16 millivolts.

On this basis, the experimental results we have obtained in the investigation of the precipitation of the 'benzoin' sol by gelatin are to be explained. With gradually increasing concentration of positively charged gelatin, there occurs a change in the charge of the 'benzoin' particles, and as the charge is reversed, there occurs the zone of precipitation. It is of great interest to note that the concentration of gelatin at which the zone of precipitation occurs depends essentially on the p_H . On the alkaline side of the isoelectric point, no precipitation occurs at all. The precipitation in fact would clearly seem to depend on the amount of positively charged gelatin present. As the p_H increases and the solution becomes more acid, an increasing proportion of the gelatin present exists as kation, and so the actual concentration at which the gelatin precipitates is decreased.

The work of Walpole [1913] is of interest. Working with an oil emulsion this observer found no precipitation with gelatin alone, but in presence of hydrochloric acid, a zone could be observed, very similar to that described with the 'benzoin' sol without hydrochloric acid. Walpole did not measure the p_H of the solutions, but gives only the normality of the acid employed. Hydrochloric acid, of normality 10^{-2} is alone sufficient to aggregate the emulsion. Between about 10^{-4} and 10^{-2} N precipitation takes place if gelatin be present in concentrations of 10^{-6} to 10^{-7} , the zone tending towards the region of more concentrated gelatin as the concentrations of acid decrease, until at about 10^{-4} N it broadens, and extends from about $10^{-4.2}$ to 10^{-6} . Our own results give a zone of the same general type, and so far confirm the findings of Walpole. In one respect, however, the experiments do not agree. Walpole finds that the line dividing the positively from the negatively charged particles in his diagram, in which the normality¹ of the acid is plotted against the concentration of the gelatin, does not coincide with the zone of precipitation. In our experiments, there was always a different charge on the particles on the two sides of the zone; both in the case of gelatin and of oxyhaemoglobin. Even the case of precipitation by ferric chloride solution (p. 642) which, similarly to gelatin precipitates at high dilutions only over a limited zone, the particles are charged oppositely at the different ends of the zone. In fact, as we have suggested above, this precipitation by ferric chloride probably bears more than a superficial resemblance to the precipitation by gelatin, as the ferric chloride is partially hydrolysed, and the ferric hydroxide exists in a colloidal condition.

Such a reaction between a lyophobic colloid and a lyophile sol is no doubt fairly general for lyophile sols of different kinds. For instance, 'benzoin' is precipitated not only by gelatin, but also by oxyhaemoglobin, serum globulin,

¹ Actually the exponents of normality and concentration respectively.

and serum albumin (see the following paper). The question arises whether the 'benzoin' sol has peculiar properties among lyophobic colloids that render it peculiarly liable to precipitation by such lyophilic sols. Reference has already been made to the oil emulsions, investigated by Walpole. It may be noted that Gann found that acid gold sols were precipitated by gelatin. On the other hand, Eggerth and Bellows [1922] in a study of the agglutination of bacteria by various proteins in presence of buffered solutions of constant salt content and varying p_H showed that the amount of protein required varied with the p_H of the solution. In the case of 'benzoin' sol, however, the conditions are so simple that it seems particularly adapted for investigation of the principles of such precipitations.

The dependence of the protective power of gelatin on the isoelectric point is less marked than that of the precipitating power, though it is fairly clearly shown in Table XIII, and is minimum at p_H 4.7, the isoelectric point of gelatin. Apparently, ionised gelatin is better able to protect than isoelectric gelatin, and this may be correlated with the greater solubility of salts in water than the undissociated molecule. For the gelatin ions, either positively or negatively charged, will probably have a greater affinity for water than the undissociated uncharged molecules, just as the benzoate ion has a greater affinity for water and is, therefore, more soluble than uncharged benzoic acid. In this way, the result may be brought into line with the views of Langmuir on the affinity of certain groups for water, and of Loeb [1923]. Loeb shows that the protective effect of gelatin is to be ascribed largely to its high affinity for water, in fact, to its high true solubility.

The results we have obtained in regard to precipitation by salts support the view that such precipitation is largely a matter of the ion of charge opposite to that on the colloidal particle. The anomalies are to be, at least largely, explained as due to the varying p_H . This is but another example of the many instances in colloid chemistry where difficulties are cleared up if due note is made of the hydrogen ion concentration.

CONCLUSIONS.

1. Precipitation of colloidal gum 'benzoin' occurs when it is added to an equal volume of gelatin of concentration about 1/500,000. The actual concentration of gelatin required varies with the p_H of the solution.

2. No precipitation occurs on the alkaline side of the isoelectric point of gelatin.

3. The charge on the particles is negative if insufficient gelatin to precipitate it has been added. If excess of gelatin has been added so that precipitation does not occur, the charge is positive. On the alkaline side of the isoelectric point of gelatin the charge is always negative.

4. Similar results are obtained with oxyhaemoglobin.

5. The power of gelatin to protect 'benzoin' against precipitation by sodium chloride depends on the p_H and is minimum at the isoelectric point.

6. Precipitation by an acid depends essentially on the concentration of hydrogen ions.

7. The amount of salt required to precipitate 'benzoin' increases with increase in the p_H . This, at least largely, accounts for the differing amounts of salts of univalent kations required to effect precipitation.

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LXXXII. THE MECHANISM OF PRECIPITATION OF COLLOIDAL GUM BENZOIN BY CEREBROSPINAL FLUID.

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IN 1920 the use of a colloidal suspension of gum benzoïn was introduced by Guillain, Laroche and Lechelle [1920] in order to detect certain pathological variations in the cerebrospinal fluid. The test is carried out by preparing in test-tubes 1 cc. of each of a series of dilutions of cerebrospinal fluid, $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{8}$, $\frac{1}{16}$, ... $\frac{1}{65,536}$, sixteen dilutions being used. To each tube is then added 1 cc. of a colloidal suspension of gum benzoïn. In our experiments we have, in order to obtain uniform suspensions, slightly modified the original instructions given by the authors of the test, thereby obtaining a very uniform 'benzoïn' sol, but one apparently rather less sensitive than the original. The sol used is fully described in the preceding paper.

Typical results obtained from fluids which are stated to give characteristic precipitations are as follows:

C.S.F. from case of General Paralysis of the Insane	344	443	444	310	000	0
" " Tabes Dorsalis	003	410	241	000	000	0
" " Meningitis (streptococcal)	000	001	444	443	100	0
" " " "	122	200	444	430	000	0
" normal*	Between 000	000	242	000	000	0
	and 000	000	000	000	000	0

The system of indicating results is that described in the preceding paper.

* Fluids from absolutely normal individuals are naturally rarely obtained, and so 'normal' denotes fluid from patients where there is every reason to believe there is no essential change in the nature of the fluid, *e.g.* measles, dementia, epilepsy.

The main point to be noticed is that the precipitation occurs in two zones. There is a normal precipitation in the seventh or eighth tube, which in pathological fluids may or may not be increased. It is particularly increased in meningitis, and usually to a less extent in general paralysis; in fact, an extension of the zone to the right often accompanies an increase of protein in the fluid, as shown by the butyric acid test [Noguchi and Moore, 1909]. The first six tubes form another zone of precipitation. Here the precipitate is particularly marked in general paralysis, and it is also notably present in the case of tabetic fluids. That is to say, in syphilis of the central nervous system well-marked precipitation occurs in these tubes. There often is also slight precipitation in cases of meningitis, but in the normal fluid there is no precipitation at all. This zone, then, appears to be affected independently of the other.

Guillain, Laroche and Lechelle showed that neither the individual proteins (obtained by fractional saturation with ammonium sulphate) nor the dialysable constituents of a fluid could reproduce the curve obtained with the original fluid. A mixture of these could, however, do so. Dible [1922] suggests that the various curves are due to variation in cerebrospinal fluids in their globulin, albumin and salt content. In view of the fact that no account was taken by these workers of the actual p_H in the various tubes it is doubtful how these results should be interpreted. It may be noted that previous workers [Cruickshank, 1920; Lange, 1912] have shown that the portion of a cerebrospinal fluid which is most effective in precipitating a gold sol is that which is precipitated by half saturation with ammonium sulphate.

In the previous paper [1923] we have shown that the precipitation of 'benzoin' by gelatin and by oxyhaemoglobin depends very greatly on the hydrogen ion concentration of the medium. At a p_H on the alkaline side of the isoelectric point, no precipitation of the sol by gelatin takes place at any concentration of gelatin. At a p_H of about 4.7, precipitation takes place when 1 cc. of the sol is added to 1 cc. of gelatin diluted 1 in 640,000. As the p_H decreases, and the solution becomes more acid, the amount of gelatin required decreases, until at a p_H of 2.8 the hydrogen ions alone are sufficient to precipitate in the absence of gelatin, and precipitation occurs until a concentration of gelatin of at least $1/640,000$ is present. Similar results have been obtained using haemoglobin. In view of the great dependence of the behaviour of proteins towards colloidal 'benzoin' on the reaction, a systematic investigation of the normal and paretic types of curves in this respect appeared to be called for. Accordingly, we have investigated the influence of reaction in the cases of serum globulin, serum albumin, and of whole serum, as well as of cerebrospinal fluids of the paretic and normal types on their behaviour as precipitants of colloidal 'benzoin.' The results explain the constancy of the zone of precipitation in the seventh and eighth tubes, and its variation in degree and not in position, and also demonstrate, we think, that the precipitation in the first five tubes (called by the authors of the test the syphilitic zone), is due, not to ordinary serum globulin or albumin, but to some extra material, which if it is protein in nature possesses a high isoelectric point, p_H between 7 and 8, and that in these cases, the actual serum globulin and albumin may be, at most, very slightly increased.

EXPERIMENTAL.

As small quantities of serum albumin and serum globulin apparently exist normally in the cerebrospinal fluid, these two proteins were first of all investigated. The mode of experiment was the same as described in the preceding paper. Fresh serum was fractionated by precipitation, first with half-saturated ammonium sulphate and then with saturated ammonium sulphate. The protein fractions obtained were washed well with ammonium sulphate solution of the requisite strength, and then transferred to a collodion sac, in which they were dialysed for 24-36 hours against tap-water. The albumin

solution was made up to the volume of the original serum and it was then ready for use. The globulin fraction was separated from the dialysed solution. It was filtered off and dissolved in sufficient sodium chloride solution (0.8 %) to make up the original bulk. If necessary it was filtered from some insoluble residue. Dilutions of these protein solutions were prepared, and also solutions of sodium hydroxide and of hydrochloric acid of various strengths. To a mixture of $\frac{1}{2}$ cc. of a globulin solution and $\frac{1}{2}$ cc. of acid or alkali was added 1 cc. of 'benzoin' sol. After shaking thoroughly, and standing overnight, the degree of precipitation was judged. The results are given in Table I, in which the dilutions given are those of the globulin or alkali after mixing, and before adding the 'benzoin.'

Table I.

Effect of p_H on precipitation by Globulin.

(The degree of precipitation is indicated by the same conventional numbers as in the preceding paper.)

		Concentration of globulin solution									
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
Concentration of sodium hydroxide	N/800	0	0	0	0	0	0	0	0	0	0
	N/1600	4 (p_H 5.3)	4	4	4	4	3	0	0	0	0
	N/3200	0	0	0	0	0	4	1	0	0	0
	N/6400	0	0	0	0	0	4	4	0	0	0
	No alkali	0	0	0	0	0	2	4	2	0	0

It will be seen that with serum globulin a zone of precipitation occurs, which, just as in the case of gelatin and of haemoglobin, alters its position on addition of alkali. In the case of globulin the last row in which precipitation occurs has the p_H 5.3. A similar experiment using serum albumin shows no precipitation in solutions of p_H greater than 4.7. That is to say, these proteins do not cause precipitation in solutions the p_H of which is on the alkaline side of the isoelectric point, i.e. in solutions in which they exist as negative ions.

Precipitation of 'benzoin' by Serum.

Experiments were carried out to determine the effect of various dilutions of serum on 'benzoin.' The results are given in Table II.

Table II.

Precipitation by Serum.

The p_H given is that of the supernatant fluid. The charges on the colloidal particles are denoted by (+) or (-).

Concentration of serum	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
	0	0	0	0	0(-)	4 (p_H 5.4)	4 (p_H 4.7)	0(+)	0
Concentration of serum	1/1024	1/2048	1/4096	1/8192	1/16384	1/32768	1/65536	1/131072	
	0	0	0(+)	4 (p_H 4.2)	4 (p_H 4.2)	2	0(-)	0(-)	

It will be seen that there are two zones of precipitation—the first is at a concentration of serum 1/8192–1/16,384, or, assuming that the total protein content of serum is 7.5 %, about a concentration of protein of 1/130,000–

1/260,000. The charge on the 'benzoin' particles, as determined by the ultra-microscopic method described in the previous paper, is negative when the concentration of protein in solution is less than that corresponding to precipitation, and positive when it is greater. This zone then apparently corresponds to the normal zone of precipitation obtained with gelatin at concentration of about 1/600,000. The second zone of precipitation occurs at much higher concentrations of serum, 1/32–1/64. The charge on the particles is positive in tube seven and negative in tube four. The p_H of the supernatant fluid in tubes five and six is 5.4 and 4.7 respectively. It is obvious that here we are encountering, at the isoelectric point of the protein, the zone of precipitation which tends to broaden and to extend into regions of high concentration. In tubes one to four the p_H is above 5.4, and so no precipitation occurs and the particles are negatively charged. As soon, however, as the p_H falls below 5.4 precipitation occurs, as some of the protein molecules (globulin) now act as bases and are positively charged and tend to precipitate the 'benzoin.' When too much positively charged protein is present, protection occurs, because too much protein is absorbed, and so in tube seven there is no precipitation and the particles are positively charged. The commencement of the zone of precipitation naturally coincides with the isoelectric point of serum globulin (p_H 5.4) as this is higher than that of serum albumin (p_H 4.7). It would appear that, as the zone of precipitation extends from p_H 5.4 to 4.7, the presence of two proteins in concentrations of the same order of magnitude tends to produce a broad zone extending between the isoelectric points of the two proteins. This is probably to be explained on the consideration that, at a p_H between the two isoelectric points, much negatively, as well as some positively charged protein will be present. In that case there will be much less tendency for the particles of 'benzoin' to acquire a positive charge, although neutralisation may quite readily occur.

It is of interest to find that a somewhat similar phenomenon may be produced if gelatin be diluted 1 in 1000 in Ringer's solution and then diluted with an equal volume of distilled water in a series of successive tubes and 'benzoin' be added. The results of such an experiment will be found in Table III.

Table III.

Precipitation by Gelatin in Ringer's Solution.

Gelatin (1 part in 1000 parts of Ringer-Tyrode solution) was prepared, and this was diluted with distilled water to the dilutions given, an equal volume of 'benzoin' sol was added to each dilution and the results read after standing overnight.

Concentration of gelatin \times 1000	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
	0	0	0	0 (-)	0 (-)	1	0 (+)	2	4	4	3½

Here two zones of precipitation appear, the first corresponding with a change in the charge on the particles from negative in high concentration to positive in low, the second with a change from positive in high concentration to negative in low. It is to be noted that Ringer's solution alone has no precipitating

effect on 'benzoin,' notwithstanding its comparatively high concentration of salt. This is because its p_H is 7.6.

Precipitation of 'benzoin' by Normal Cerebrospinal Fluid.

A normal cerebrospinal fluid was examined in regard to precipitation of 'benzoin' and the charges on the particles in the unprecipitated zones. The curve obtained was as follows:

000, 000, 320, 000, 000, 0.

Precipitation of about the usual amount was seen in the seventh and eighth tubes. On investigating the reaction in tube 7 this was found to be equivalent to p_H 4.5. It was, however, interesting to find that the charges on the particles of 'benzoin' were negative on either side of the zone of precipitation. The explanation is evident from the following experiment which shows the effect of the addition of acid or alkali to another normal fluid.

Table IV.

Effect of p_H on precipitation by normal C.S.F.

$\frac{1}{2}$ cc. of diluted C.S.F. and of acid or alkali added to each tube and then 1 cc. of 'benzoin' solution. Reading after standing overnight.

		Concentration of Cerebrospinal Fluid (Normal)													
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096		
Concen- tration of hydro- chloric acid	N/400	0	0	0	—	—	—	—	—	—	—	—	—	—	—
	N/800	0	0	0	0	—	—	—	—	—	—	—	—	—	—
	N/1600	0	0	0	0	0	0	—	—	—	—	—	—	—	—
	N/6400	0	0	0	0	0	0	0	0	—	—	—	—	—	—
	Distilled water	0	0	0	0	0	1	0	0	0	0	—	—	—	—
Concen- tration of sodium hydroxide	N/6400	0	0	0	0	0	3 (p_H 4.4)	1	0	0 (-)	0	0	0	0	0
	N/1600	0	0	0	0	4 (p_H 4.6)	4	4 (p_H 3.4)	1	0	0	0	0	0	0
	N/800	0	0	0 (-)	4 (p_H 4.7)	4 (p_H 3.4)	4 (p_H 3.3)	4	4 (p_H 3.0)	3	3	2	0	0	0
	N/400	0	0 (-)	4 (p_H 4.7)	0 (+)	0	4 (p_H 3)	4 (p_H 3)	4 (p_H 2.8)	4	4	4	4	4	4

Fig. 1 shows the resulting zone of precipitation if the concentration of fluid be plotted against the p_H of the tubes showing precipitation.

Here it will be seen that the added acid or alkali had a marked effect on the position of the zone of precipitation. This zone varies in a similar fashion to a zone of precipitation obtained with a protein. It does not extend to tubes which have a p_H greater than 4.7. This corresponds to the isoelectric point of serum albumin.

It will be evident from the table and figure that in a normal c.s.f. test, the zone of precipitation is not really crossed at all. It is entered and left again, as shown by the dotted line in the figure, which indicates approximately the p_H of the liquid in the various tubes after the 'benzoin' has been added, but where neither acid nor alkali is added. The result is that the 'benzoin' particles on both sides of the zone of precipitation are negatively charged since the positive region is never entered.

The reason for the constancy of position of the zones of precipitation obtained with normal fluids is evident. In the tubes in the first column, the amount of c.s.f. present is high and so there is considerable buffering of the

solution. The result is that after addition of the 'benzoin' the p_H remains high and it is not until the seventh or eighth tube that the acidity of the 'benzoin' is sufficient to lower the p_H to about 4.7. At this point the protein will have

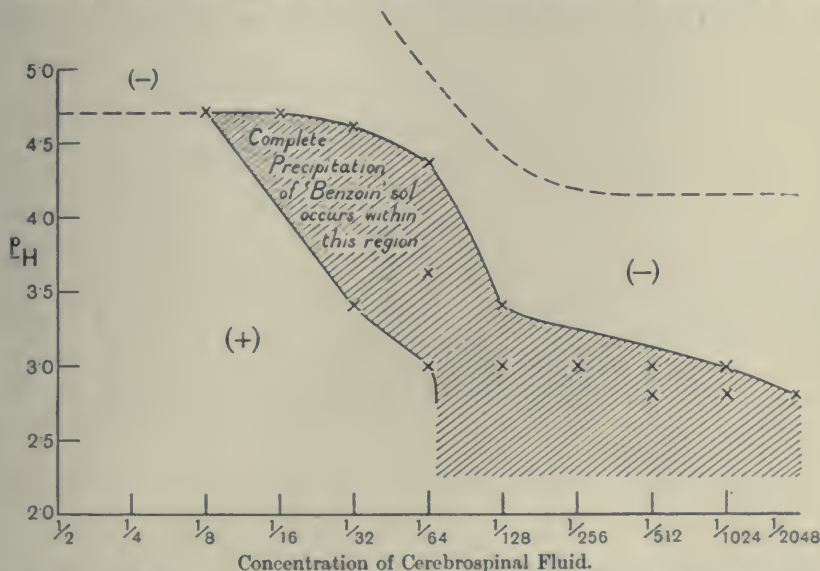


Fig. 1. Precipitation of 'benzoin' by normal cerebrospinal fluid as influenced by the p_H . The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added. As will be seen, the region of complete precipitation is not entered, the maximum precipitation being of the order 1/2. In other cases the line may enter and leave but does not cross the zone of complete precipitation. (+) and (-) refer to the charge on the 'benzoin' particles in the different regions.

lost its negative charge and so will begin to act as a precipitant of the 'benzoin.' Naturally, owing to slight variations in amount of buffering of the solution (CO_2 content, etc.), and possibly in the amount of protein the exact p_H in the seventh and eighth tubes will vary slightly; hence the slight variations in the amount of precipitation produced at this point even with normal cerebrospinal fluids. As we have remarked above, with some there is no precipitation at all. It is clear that this will occur where the protein content is low, or the degree of buffering is high. On the other hand, it is clear that in fluids, e.g. from a case of meningitis, where the protein content is much in excess, the zone will be extended into regions of higher dilution.

Precipitation of 'Benzoin' by a Cerebrospinal Fluid from a case of General Paralysis.

In the following experiment to determine the effect of acid and alkali on the zone of precipitation, a typical fluid from a case of general paralysis giving the following curve was used:

444, 443, 443, 100, 000, 0.

The essential point of difference between this and the normal curve is that complete precipitation has occurred in the first five tubes.

The details of the experiment recorded are the same as in the case of the normal fluid.

The result is shown in Table V.

Table V.

Effect of p_H on precipitation by Paretic C.S.F.

Concentration of Cerebrospinal Fluid (G.P.I.).

	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Concentration of sodium hydroxide	N/100	—	0	0	0	0	0	0	0	0	0
	N/200	—	0	0	0	0	0	0	0	0	0
	N/400	3 (p_H 7.8)	0	0	0	0	0	0	0	0	0
	N/800	4 (p_H 7.6)	0	0	0	0	0	0	0	0	0
	N/1600	4 (p_H 7.8)	4 (p_H 7.5)	3	0	0	0	0	0	0	0
	N/12800	4 (p_H 7.6)	4 (p_H 7.2)	4 (p_H 6.8)	0 (—)	4 (p_H 5.1)	2	0	0	0	0
	Distilled water	4 (p_H 7.3)	4 (p_H 7)	4 (p_H 6.8)	3	4 (p_H 5)	3 (p_H 4.4)	0	0	0	0
Concentration of hydrochloric acid	N/6400	—	4 (p_H 7.2)	4 (p_H 7)	4 (p_H 5)	4 (p_H 4.6)	4 (p_H 4.1)	3	0	0	0
	N/1600	—	4 (p_H 7)	4 (p_H 6.6)	0 (+)	0	4 (p_H 3.8)	4 (p_H 3.6)	3 (p_H 3.4)	0	0
	N/1200	4 (p_H 7.4)	4 (p_H 6.9)	4 (p_H 5.4)	—	—	—	—	—	—	—
	N/800	4	4 (p_H 6.9)	0	—	—	—	—	—	—	—
	N/600	4	4 (p_H 6.2)	0	—	—	—	—	—	—	—
	N/400	4 (p_H 7)	4 (p_H 5.5)	0	0	0	0 (+)	4 (p_H 3)	4	4	4
	N/300	4 (p_H 7)	0	0	—	—	—	—	—	—	—
	N/200	4 (p_H 5.9)	0	0	0	0	0	4 (p_H 2.6)	4	4	4
	N/100	0	0	0	0	0	0	4 (p_H 2.4)	4	4	4

Fig. 2 shows the zone of precipitation if the p_H be plotted against the concentration of fluid in tubes showing precipitation.

It will be seen that up to a p_H of about 5 the curve is very similar to that given by a normal fluid. Notwithstanding the increase in total protein content, as shown by the Noguchi reaction, the zone of precipitation at p_H 4 occurs at a concentration of about 1/64 just as with the normal fluid. It would appear from this that there is no definite increase in the amount of serum globulin or albumin present, because if this were increased one would expect that the zone would be shifted to the right. On the other hand, precipitation is seen to occur in a region between p_H 5 and p_H 7 to 8. This is a zone non-existent with a normal fluid, serum globulin or serum albumin and so presumably the substance which causes this precipitation is not present in these.

If it is a protein, and the marked increase in the Noguchi reaction in most cases of general paralysis might suggest this, it is presumably a protein of isoelectric point p_H 8, since the 'benzoin' is negatively charged and proteins, as far as our experiments go, precipitate it only when they are at, or on the acid side of, their isoelectric point. They fail to do so when they are negatively charged. At p_H 7 the normal proteins (serum albumin and serum globulin) are negatively charged and it is difficult to understand how they could be the active agents.

It may be noted that precipitation occurs over a continuous area. This might at first sight appear rather inexplicable, as instead two zones might rather be expected. However, the explanation is, no doubt, similar to that of the existence of a zone with normal serum extending approximately from

p_H 5.4 to 4.7, that is, between the isoelectric points of serum globulin and of serum albumin. If two proteins are present in comparable concentrations, then at a p_H between the two isoelectric points, the positively charged particles of the protein with the higher isoelectric point will neutralise the negative

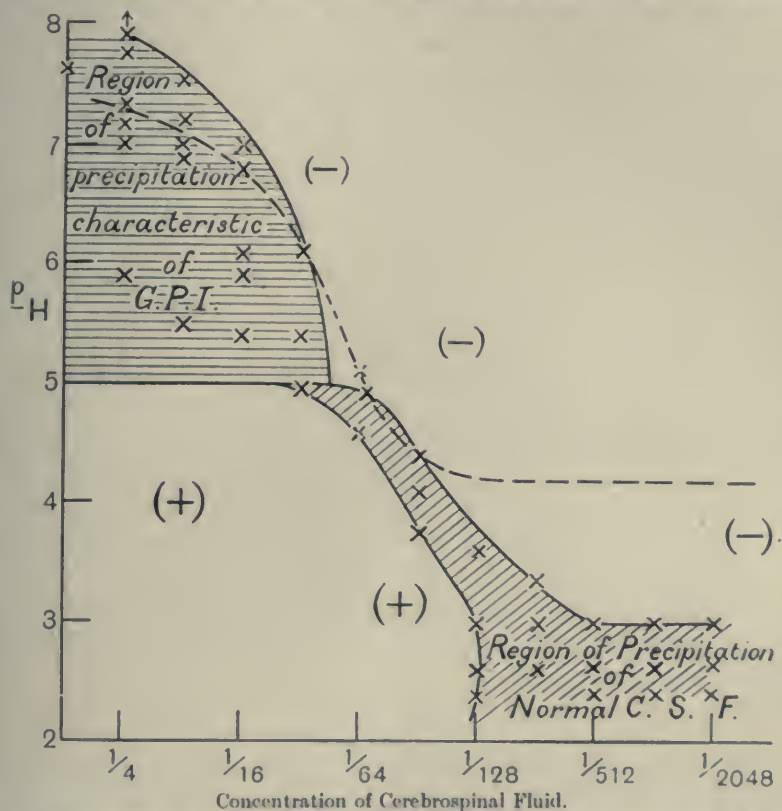


Fig. 2. Precipitation of 'benzoin' by cerebrospinal fluid from patient with G.P.I. The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added.

(+) and (-) refer to the change in the 'benzoin' particles.

charge on the colloidal 'benzoin' particles, but it is doubtful whether it will confer a positive change on them, as the other protein in solution is still completely negative, and so will tend to prevent the positive protein being absorbed on the particles.

DISCUSSION.

Goebel [1921] has made the observation that in the case of the mastic reaction, on diluting cerebrospinal fluid from a case of general paralysis with a normal fluid, the curves obtained are still essentially of the paretic type. He further suggests that there are two fundamental types of curve—the meningeal type indicating increased permeability of the vessels and consequent increase in serum protein in the fluid, and the paretic type characteristic of conditions associated with tissue destruction. Lange [1912] would appear

to suggest that in paretic fluids evidence of the presence of nucleoprotein may be obtained. Cruickshank [1920] suggests that in general paralysis there is a change in the ratio of globulin to albumin in the cerebrospinal fluid and as the globulins from a normal fluid do not give the reaction even on concentration, this is associated with an alteration in the electrical charge on the globulins.

Our own results confirm the view that there is a qualitative difference between the paretic cerebrospinal fluid and the normal, as in the table showing precipitation by a paretic fluid the zone due to the normal constituents can be identified and differentiated from the peculiar and characteristic abnormal zone. The essential feature of the latter is that it occurs between p_H 5.5 and 7.5, and it is clear that no interpretation or explanation of a curve can be made without taking into account the reaction of the solution in which precipitation occurs. This, of course, is in accordance with the fact that the physico-chemical behaviour of proteins is a function of the reaction, more particularly of the reaction relative to their isoelectric point.

The general effect of alkalinity on the colloidal reactions of the cerebrospinal fluid has already been noted in the literature. For instance, Bannerman [1919] has shown that the globulin fraction of a paretic fluid can reproduce the paretic curve of gold, if it is made slightly alkaline, and Presser and Weintraub [1922] and Sahlgren [1922] attributed the non-precipitation of mastic by high concentrations of normal cerebrospinal fluid to the protective action of the alkali present. But the precipitation of a colloid is determined by the two factors protein and reaction together, and these cannot be considered independently, for it is the electrical condition of the protein that is of importance.

CONCLUSIONS.

1. The zone of precipitation occurring in normal and pathological cerebrospinal fluids in the seventh and eighth tubes in the colloidal 'benzoin' test is due to precipitation of the negatively charged 'benzoin' particle by the positively charged protein of the fluid.

2. The constancy of its position depends on the fact that the p_H must be below 4.7 before any such precipitation can occur. The variations in the degree of precipitation and in the number of the tubes included in the zone, depends on the relative amount of protein present and the degree of buffering of the fluid.

3. The precipitation occurring in the first five tubes in fluids from cases of general paralysis of the insane is due to the presence of some abnormal constituent, and not to an increased amount of the proteins responsible for the normal precipitation.

4. The general dependence of precipitation by cerebrospinal fluid on the hydrogen ion concentration has been investigated.

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LXXXIII. OBSERVATIONS ON THE SOLUBILITY OF INSULIN.

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(Received July 10th, 1923.)

It is of the greatest importance for the isolation and purification of insulin to know its solubility in different organic solvents. Our knowledge as to this has been up till now very limited. Insulin is freely soluble in water. We know, further, that it is insoluble in ethyl ether, toluene and neutral ethyl alcohol. Doisy, Somogyi and Shaffer [1922] find that insulin is certainly soluble in water, but that it is incompletely precipitated from its solution at p_H about 5 to 6. They find, also, that it is soluble in "alcohol" except at its isoelectric point.

The following experiments on the solubility of insulin have been performed with "crude insulin" prepared partly according to Collip [1922] and partly according to Shaffer. About 20–25 mg. contained one "rabbit-unit" and 0.04–0.02 mg. produced characteristic convulsions in mice.

The solubility experiments were made in the following way. It might be expected that the salt of insulin would have a different solubility from the free compound. For this reason we have made the experiments in two different series: acid and alkaline extractions.

Extractions at acid reaction. 10 mg. insulin were dissolved in 1 cc. water and 1 cc. acid buffer solution (equal parts of *N* acetic acid and *N* Na-acetate, p_H about 4.75) was added. The solution was equally distributed (0.2 cc.) in ten small Petri dishes and evaporated to dryness in a vacuum exsiccator over sulphuric acid. The activity of the insulin was not hereby diminished. 2–3 cc. of the organic solvent were now added. The solvent was filtered from the residuum and again evaporated in a dish. 2 cc. of physiological salt solution were added and 0.5 cc. of this solution was injected into each mouse. The animals were placed in a thermostat at 28° and the occurrence of convulsions was noted. The convulsions demonstrated the solubility of the insulin in the solvent used. The activity of the residuum from the extractions was often controlled in the same way in order to show that the insulin had not been destroyed during the operations.

The extraction at alkaline reaction was made in the same way only with the difference that an alkaline buffer was used (sodium hydroxide-borâté, p_H 10) instead of the acid.

With this method we tried some different organic liquids until we found in absolute methyl alcohol the first water-free solvent.

Extraction at acid reaction.

Solvent	Extract	Residuum
Tetrachloromethane	- - - -	+ + + +
Ethyl acetate	- - -	+ + - -
Light petroleum	- - -	+ - -
Methyl alcohol	+ + (+)	+ + -

The plus sign indicates that convulsions occurred, the minus sign that they failed to appear. With tetrachloromethane, ethyl acetate and light petroleum no effect on the animals could be observed. This means that, inasmuch as the quantity of insulin used was many times greater than that which produces convulsions, the insulin is insoluble or only very slightly soluble in the solvents mentioned. The methyl alcohol on the contrary dissolves the insulin, although the extraction is incomplete.

Extraction at alkaline reaction.

Solvent	Extract	Residuum
Tetrachloromethane	- - -	+ + +
Ethyl acetate	- - -	
Ethyl ether	- - -	
Ethyl alcohol	- - -	
Chloroform	- - -	
Acetone	- - -	
Light petroleum B.P. below 50°	- - -	
" B.P. 60-80°	- - -	
Methyl alcohol	+ + (+)	+ + +

Here, too, we obtain the same results. Methyl alcohol alone is able to extract the insulin.

The solubility experiments with methyl alcohol were repeated on a larger scale. Neutral, anhydrous methyl alcohol dissolves the insulin only with difficulty. Acid methyl alcohol dissolves it much more easily. The pure alcohol was acidified by passing in dry HCl. 200 cc. of this liquid, containing 6.6 millimols HCl dissolved 500 mg. crude insulin only leaving a slight residue.

The insulin may be precipitated from the methyl alcoholic solution by reagents which mix with methyl alcohol, but in which the insulin itself is insoluble. This procedure was used in order to detect other solvents for insulin. A saturated solution of insulin in pure, neutral methyl alcohol was prepared. The solution was distributed in a series of test-tubes and several volumes of the different fluids were added to each of the tubes. A precipitation occurred with the following fluids: ethyl acetate, ethyl isobutyl, and amyl alcohol, chloroform, tetrachloromethane, acetone, light petroleum, ethyl ether, benzene, xylene and pyridine. Whether the precipitates contained insulin was tested by activity experiments on mice, positive results being obtained with those from ethyl acetate, ethyl, isobutyl, and amyl alcohol, ethyl ether, light petroleum, acetone and tetrachloromethane. These experiments thus show the insolubility of the insulin in these solvents.

By the addition of glacial acetic acid, phenol, formamide and glycerol to the methyl alcoholic solution no precipitations were obtained. The solubility in glycerol can be explained through the high water content of this solvent.

But anhydrous glacial acetic acid, phenol and especially formamide were found capable of dissolving considerable amounts of insulin. It could be precipitated from these solutions, for instance, by ethyl ether, and this precipitate was very active.

These facts concerning the solubility of the insulin may be of value for further research on the nature of this hormone. They do not say much about its chemical nature, but I will remark that methyl alcohol, phenol and above all formamide are solvents for albumoses. Other facts also favour the belief that insulin belongs to this class:

1. Crude insulin gives a decidedly purple colour by the biuret test, and an orange colour by the xanthoproteic test.
2. It gives a precipitate with nitric acid, which disappears on heating.
3. It is not precipitated on boiling.
4. It is precipitated from watery solution by half saturation with ammonium sulphate.
5. It is very easily destroyed by trypsin.
6. It is impossible to demonstrate any dialysis of insulin through parchment membranes. It cannot be ultrafiltered through 3 % collodion filters.
7. It is freely soluble in pure, salt-free water.

I cannot find any valid reason for the suggestion that insulin is a globulin.

SUMMARY.

1. Insulin is insoluble or very slightly soluble in tetrachloromethane, ethyl acetate, ethyl alcohol, isobutyl alcohol, amyl alcohol, chloroform, acetone, light petroleum, ethyl ether, benzene, xylene and pyridine. Easily soluble in methyl alcohol, glacial acetic acid, phenol and formamide.
2. Several facts argue in favour of its being an albumose.

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LXXXIV. OBSERVATIONS ON CERTAIN REDUCING AND OXIDISING REACTIONS IN MILK.

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(Received July 16th, 1923.)

I. THE REDUCTION OF NITRATE TO NITRITE.

IN 1904 Kastle and Elvove [1904] first described the reducing action of a substance contained in the sap of the potato tuber upon sodium nitrate in the presence of acetaldehyde and other less potent activators. During the course of an investigation of this and similar reducing reactions of certain plant extracts, comparative experiments were made with cow's milk, since this substance has long been known to exert a reducing action upon methylene blue, both in the presence¹ and in the absence of aldehydes. It was then found that milk also possesses the property of reducing nitrate to nitrite in the presence of acetaldehyde, but it was subsequently ascertained that this observation had been previously recorded by Bach [1911] who had concluded that the action was due to an enzyme which appeared to be present in very small quantity. The experimental evidence here brought forward does not support this view. Later Bach [1912] found that a similar reducing reaction is given by an extract of calves' liver, an observation which was subsequently made anew by Harris and Creighton [1912].

The reduction of sodium nitrate to sodium nitrite by milk is easily demonstrated as follows:

Exp. 1. To 10 cc. of fresh cow's milk, 3 cc. of 4 % sodium nitrate are added, together with two drops of 10 % acetaldehyde; the tube is placed in a thermostat at 50° for ten minutes, then removed, cooled under the tap, and tested for nitrite by the addition of a few drops of Griess-Ilosvay reagent. If nitrite be present a pink colour will appear very quickly and rapidly increase to its maximum intensity, whilst a control in which the nitrate solution is replaced by an equivalent volume of water gives a negative reaction. The intensity of the colour varies with the amount of nitrite present; the normal range is from pale pink to magenta; if in excess, the magenta colour quickly becomes brown.

¹ Scharlinger reductase.

Although acetaldehyde is not essential for the process, it is requisite if time is a factor of any importance, since the amount of nitrite produced in its absence is very small even after some hours, and in dealing with milk, with its generally large bacterial flora, the sooner an experiment is completed the better; antiseptics such as toluene, lysol, and chloroform may be employed in prolonged experiments.

Acetaldehyde may be replaced by other aldehydes such as benzaldehyde and cinnamaldehyde; formaldehyde, however, in our experience has a retarding and sometimes an inhibiting action.

The reducing agent is destroyed by boiling, the critical temperature being about 72°, but much depends upon the time of exposure to the high temperature, a fact already commented on by previous authors for enzymes of milk, Schardinger's reductase for instance [Rullmann, 1913, 1914; Seligmann, 1906]. The following typical observations show the importance of the time factor and also that the Schardinger reductase is more sensitive to heat than is the nitrate-reducing agent.

Table I.

Temp. ° C.	Time in minutes	Schardinger reductase	Nitrate reducing agent
70°	17	+	+
72°	5	+	+
	10	+	+ weak
	15	-	+ very weak
75°	15	-	+ very weak
80°	10	-	-

With the object of preparing from milk a clear solution containing the nitrate-reducing agent freed from fat or protein, the milk was coagulated by various means, but after separation of the curd, produced either by excess of acetic or lactic acid or by rennin, the whey no longer reduces nitrate to nitrite. Saturation with ammonium sulphate or the addition of basic lead acetate (6 cc. to 100 cc. of milk¹) likewise completely removes the nitrate-reducing agent with the proteins, the clear whey then giving negative results. It is this fact which makes the separation of the active principle from milk a matter of considerable difficulty and attempts to do this have so far not met with success.

With regard to the measurement of the activity of the nitrate-reducing agent, the fact that milk contains much protein renders the estimation of small amounts of nitrite a matter of no little difficulty. Many methods were tried and the one finally adopted as giving the most reliable results was as follows. To the volume of milk an equal volume of a saturated solution of ammonium sulphate was added, the clot was filtered off and the whey saturated with ammonium sulphate by the addition of an adequate quantity of the crystalline salt. The mixture was then again filtered through a folded filter paper. The nitrite contained in the filtrate was estimated colorimetrically by means of metaphenylene diamine. On theoretical grounds this procedure

¹ It is well to centrifuge off the fats first.

may be open to question in view of the possible interaction between the ammonium sulphate and the sodium nitrite: it was found, however, that in the time spent in salting out the proteins at the room temperature at which this operation was carried out, the loss of nitrite from this cause was, if any, within the margin of error.

The following illustrates a typical experiment on the reduction of nitrate by milk.

Exp. 2. 450 cc. of milk, contained in a stoppered bottle, were mixed with 30 cc. of 4 % sodium nitrate and 6 cc. of 10 % acetaldehyde. After a thorough shaking, the mixture was left at rest in a thermostat at 45° and only shaken up regularly every 15 minutes when taking a sample for estimation. The experimental results obtained are shown in the form of a curve (Fig. 1).

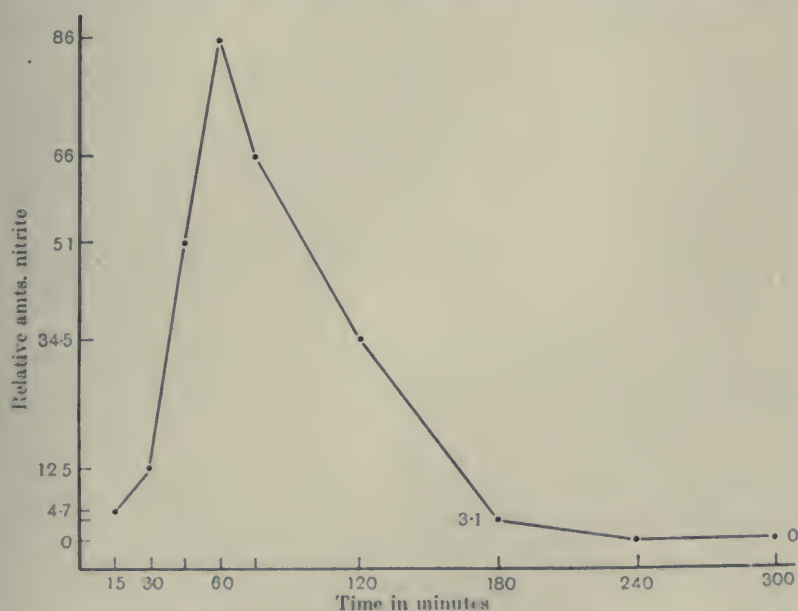


Fig. 1.

This curve is typical for the conditions named, of which the most important is the amount of shaking, since this not only preserves the homogeneity of the fluid but also ensures uniform aeration. The significance of aeration will later be apparent (p. 675).

The curve is peculiar and merits some consideration. If the reduction of the nitrate is a simple enzymic reaction, the curve should ascend until the whole of the nitrate is reduced to nitrite; if the agent is a reducing substance but not an enzyme, the curve should ascend until the whole of this substance is used up. In neither case should there be a fall to zero and it therefore becomes necessary to account for the disappearance of the nitrite. It is unreasonable to suppose that the same agent which reduces the nitrate,

should suddenly break off its action on the nitrate, when the latter is still in excess, and initiate an attack on the nitrite. Even if this were so (provided the agent is enzymic or, if non-enzymic, is in sufficient abundance) the curve should ascend again, since in the experiment there was nitrate sufficient to produce more nitrite. On the assumption that the system is a single agent capable of reducing both nitrate and nitrite, the ultimate result should be that some nitrite should remain just so long as there is an excess of nitrate present, but, the ultimate result should not be zero until all the nitrate has disappeared. The fact that the nitrite ultimately disappears, although excess of nitrate remains, strongly indicates that the mechanism responsible for the production of nitrite has ceased its activity, wherefore it probably is not an enzyme but rather a reducing substance present in limited quantity. Moreover, if the two reductions were really brought about by one and the same substance, whether enzymic or not, the final product of the combined actions alone should be detectable, since the nitrite produced by the first reduction would be in the most favourable condition for immediate further reduction and so would have no opportunity for accumulation to a maximum as is actually the case. These considerations indicate strongly the presence of two active substances; the one reducing nitrate to nitrite, for which we propose the term "atite¹" the other converting the nitrite into something else. Considerations such as these led to much experimentation which in time yielded evidence which proved the existence of an agent, other than atite (p. 678). Before dealing with this it may be mentioned that on the assumption that the nitrite itself was further reduced, milk was quantitatively examined for ammonia before and after treatment. To do this, increasing quantities of sodium nitrite were added to a series of tubes containing milk and a little acetaldehyde (1 cc. of 10 % aldehyde to 75 cc. of milk) placed in a thermostat at 45°. A small quantity of the mixture from each tube was periodically tested and that tube from which most nitrite had disappeared was selected for estimation as having, presumably, most ammonia. The details of the procedure followed may be omitted since the result showed that the quantity of ammonia present after the destruction of nitrite was no greater than that found in the original milk.

In view of the presence in milk of a reductase capable of reducing methylene blue directly, and also an indirect reductase (Schardinger enzyme) only capable of bringing about this reduction in the presence of formaldehyde [see Lane-Claypon, 1913; Harden and Lane-Claypon, 1912], the relationship between the nitrate reducing agent and the Schardinger reducing enzyme must be inquired into.

That the nitrate reducing agent of the potato is not identical with the Schardinger enzyme is indicated by the following experiment.

Exp. 3. Four tubes are made up and treated as follows:—(1) 1 g. of potato tuber is ground in a mortar under 10 cc. of 4 % sodium nitrate; the

¹ A convenient term since it indicates the conversion of nitrate to nitrite.

mash is placed in a test tube and the temperature raised to 57° in a thermostat; three drops of 10 % acetaldehyde are then added. (2) The same preparation, but the sodium nitrate is replaced by three drops of a 1 % aqueous solution of methylene blue. (3) 5 cc. of milk are mixed with 5 cc. of 4 % sodium nitrate and then as for the first tube. (4) The same preparation as (3), but three drops of methylene blue in place of the sodium nitrate. The four tubes, together with two controls lacking sodium nitrate, are then placed in a thermostat at 57° ; numbers (1) and (3) and the controls are removed after two or three minutes and tested for nitrite with Griess-Ilosvay reagent, whilst numbers (2) and (4) are allowed to remain for a time sufficient to give a result. Typical reactions are as follows:

Potato	{ 1. Griess-Ilosvay +	Control -
	{ 2. Schardinger -	
Cow's milk	{ 3. Griess-Ilosvay +	Control -
	{ 4. Schardinger +	

It may be argued that the negative result with methylene blue is due to the conditions of the experiment: this may be true; but without considering in any detail the botanical aspects of the question, which consideration is reserved for a future occasion, it may be stated that in no instance has success in reducing methylene blue with the sap of various plants been achieved under conditions which invariably gave positive reactions with milk.

II. OXIDATION OF NITRITE TO NITRATE.

On p. 673 allusion has been made to the significance of shaking, and, in consequence, of aeration. This is well brought out in the following experiments:

Exp. 4. A mixture of 500 cc. milk, 6.6 cc. of 10 % acetaldehyde, and 33.3 cc. of sodium nitrate was placed in a glass container of 1 litre capacity which was throughout the experiment rotated in the thermostat at 45° to ensure continuous agitation and aeration. A precisely similar mixture contained in a vessel of the same capacity was placed in the thermostat but not rotated and only shaken every 15 minutes. Samples from each were periodically taken and the amount of nitrite estimated.

Table II.

Time in minutes	Relative amount of nitrite		Time in minutes	Relative amount of nitrite	
	Continuously rotated	Periodically shaken		Continuously rotated	Periodically shaken
15	0	0.04	90	0	0.14
30	0	0.11	120	0	0
45	0	0.22	180	0	0
60	0	0.52	240	0	0
75	0	0.55			

The results indicate that with thorough aeration no measurable amounts of nitrite are detectable, whereas, when the aeration is limited, quantities of

nitrite rising to a maximum and falling off again are produced as in the experiment represented by Fig. 1. A further test was applied by conducting two experiments side by side, the only difference being that the one lacked oxygen.

Exp. 5. 300 cc. of milk together with 20 cc. of 4 % sodium nitrate were placed in a bottle fitted with a cork through which passed a delivery tube reaching to the bottom and an exit tube; the air was exhausted from the bottle and a rapid stream of nitrogen was then bubbled through for about half-an-hour, to wash out the last traces of air. 4 cc. of acetaldehyde were thereupon rapidly added and nitrogen was again bubbled through. The vessel was then closed and placed in the rotating mechanism of the thermostat at 45°. A similar mixture of aerated milk, nitrate and aldehyde was placed in a bottle of suitable capacity and fixed in the rotating mechanism of the same thermostat. Both bottles were thus equally agitated throughout the experiment. Samples from each were periodically removed, without admitting air to the anaerobic tube, and the nitrite estimated, with the following results:

Table III.

Time in minutes	Relative amount of nitrite		Time in minutes	Relative amount of nitrite	
	Aerobic conditions	Anaerobic conditions		Aerobic conditions	Anaerobic conditions
30	0	0.024	120	0	1.06
45	0	0.125	180	0	1.18
60	0	0.461	240	0	1.30
75	0	0.80			

The figures in Table III representing the relative amounts of nitrite produced under anaerobic conditions show a gradual falling off, but the duration of the experiment was insufficient to show whether there was a limit to the amount of nitrite which could be produced. For this reason an experiment similar in all respects to the foregoing, with the exception that hydrogen was used instead of nitrogen, was carried out for a longer period. The results are set out in Table IV and Fig. 2.

Table IV.

Time in hours	Relative amount of nitrite	Time in hours	Relative amount of nitrite
1	0.74	5	2.67
2	1.47	7	2.90
3	2.03	8	2.89
4	2.30	9	2.89

From these and like experiments it is clear

(1) That the action of atite is best manifested in the absence of oxygen which element is not a requisite, in that the action of atite is a reductive process.

(2) That the estimations of nitrite obtained in an atite experiment, Fig. 1 for example, represent the balances between the formation and destruction

of nitrite in a given time under the experimental conditions, of which the degree of aeration is of the greatest importance.

(3) That nitrite is present in limited amount and can only reduce a certain quantity of nitrate.

(4) That the destruction of nitrite, since it is so rapidly effected in a free supply of oxygen, is an oxidative process rather than a reductive process, as was first supposed. This latter conclusion was proved to be correct by the following experiment in which the production of nitrate was established.

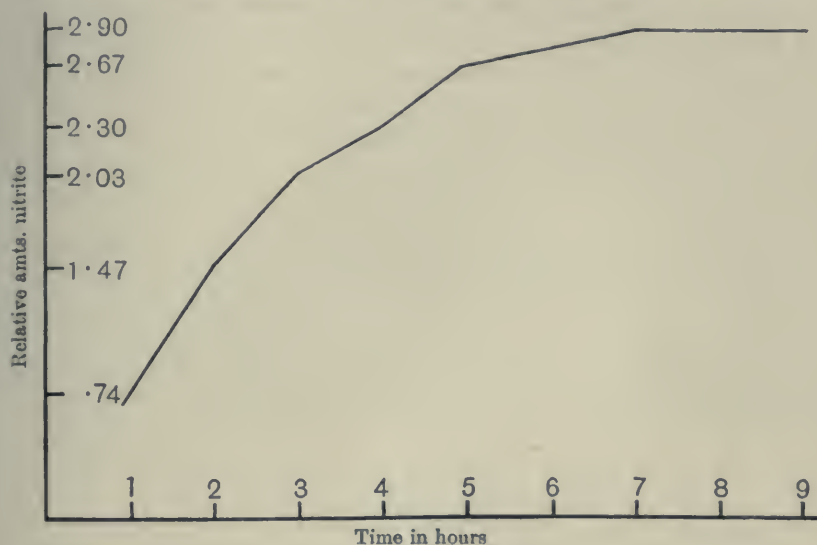


Fig. 2.

Exp. 6. A number of tubes were prepared each containing milk and aldehyde in the proportion of 75 cc. of milk to 1 cc. of 10 % aldehyde; to each tube was added a standard solution of sodium nitrite in serially increasing amounts. The tubes were then rotated in the thermostat at 45° and the contents tested with Griess-Ilosvay reagent at definite intervals of time for nitrite. The accompanying table, from which the lower additions of nitrite have been omitted, sets out the results.

Table V.

Time in minutes	Cc. of standard sodium nitrite added to each tube ¹												
	40	45	50	55	60	65	70	75	80	85	90	95	100
30	-	-	+	+	+	+	+
60	.	.	-	-	-	-	-	+	+	+	+	+	+
90	-	+	+	+	+	+
120	-	-	+	+	+
150	-	+	+
210	-	+
390	+

¹ In order to prevent undue dilution a stronger solution of nitrite was actually employed for these experiments so that the actual volumes of liquid added were only 1/10 of those given in these columns.

The tube to which 10 cc. of strong nitrite equivalent to 100 cc. of standard sodium nitrite had been added when tested after $6\frac{1}{2}$ hours with Griess-Ilosvay reagent was found to give no reaction, indicating absence of nitrite. In order to ensure absence of traces of nitrite the solution was further warmed with pure nitrate-free urea and hydrochloric acid; it was then tested for nitrate with diphenylamine when a very strong positive reaction was given; this observation has been several times confirmed and the conclusion is therefore reached that the nitrite formed by the action of atite is reconverted back into nitrate. Furthermore this reversion is effected by some agent contained in the milk, since controls to test the action of atmospheric oxygen and of acetaldehyde upon sodium nitrite in the absence of milk gave negative results. The facts set forth in Table V indicate that this agent, which is responsible for the oxidation of nitrite to nitrate and for which the name of *ilate*¹ is proposed, is present in milk in limited amount, the quantity present in 75 cc. of milk being unable to effect the oxidation of 110 cc. of 0.182 % standard nitrite even after $6\frac{1}{2}$ hours. As has been seen, it is only active in the presence of free oxygen and like atite it is dependent on some accelerator or activator such as acetaldehyde (see p. 679). Like atite it is precipitated with the proteins when milk is coagulated and is destroyed in five minutes at a temperature of 99°, in fact its thermolability is similar to that of atite, the thermal inactivation point being between 70 and 75° (Table VI).

Table VI.

Temp. ° C.	Reaction with Griess-Ilosvay reagent after the stated intervals of time		
	5 minutes	10 minutes	15 minutes
80	+	+	+
75	+	+	+
70	+ weak	+ weak	+ weak
65	+ weak	+ weak	+ very weak

After these observations had been made, it was found, on referring to the literature, that Bach [1911] had also noticed in his experiments on milk a tendency for the nitrite to disappear during the course of the reaction; he was however unable to find a reason for this.

It is well known that peroxidase is described as occurring in milk [for literature see Lane-Claypon, 1913]. The question arises whether the oxidation of nitrite to nitrate is not due to the activity of this peroxidase, or, in other words, whether itate and peroxidase are identical. Experimental evidence goes to show that there is undoubtedly a close connection between the two: invariably has it been observed that the disappearance of the itate activity is coincident with the disappearance of the positive guaiacum reaction and *vice versa*, and conditions which destroy the one will destroy the other. If then itate is identical with milk peroxidase it would at any rate appear doubtful whether it is enzymic in nature, since it is used up in the course of its activity.

¹ Since it converts nitrite into nitrate.

ERRATA

Vol. 17 p. 678, lines 1-2, for "standard sodium nitrite"

read "0.0182 % sodium nitrite"

line 15, for "110 cc. of 0.182 % standard nitrite"

read "11 cc. of 0.182 % sodium nitrite"

Furthermore, since it was observed that milk which gives a positive reaction with guaiacum and hydrogen peroxide is unable to oxidise nitrite in the absence of acetaldehyde, even if oxygen be present, a comparative experiment was set up using the strong direct oxidase of the potato. But in this case also it was found that no oxidising action was exerted upon added nitrite.

Also it has been found that neither the oxidase of the potato tuber nor the peroxidase of horse-radish is destroyed in the presence of acetaldehyde or of nitrite, whilst the peroxidase reaction of milk is so destroyed under the same experimental conditions. From these facts it is concluded that the so-called peroxidase of milk at any rate differs materially in its properties both from a typical peroxidase and from a typical oxidase of plant origin.

III. THE ACTION OF ACETALDEHYDE.

Acetaldehyde, or some comparable substance, is either requisite, or is necessary as an accelerator, for the activity of atite, itate and Schardinger reductase. It is, however, peculiar in the fact that it will destroy atite, itate and Schardinger enzyme in the presence of oxygen, which leads to the paradox that the same substance which activates also destroys. The following experiment illustrates this.

Exp. 7. Tubes of milk with added acetaldehyde and sodium nitrite in the proportion of 1 cc. of 10 % aldehyde and 4 cc. of a standard nitrite solution (of ten times the usual strength) to 75 cc. milk were rotated in the thermostat at 45°. After 25 minutes they were removed and tested:

Table VII.

Mixture	Guaiacum	Griess	Methylene blue
Milk alone	+	.	+
" and aldehyde	-	.	-
" " nitrite	+	+	+
" " nitrite and aldehyde	-	-	-

The results show that heat alone does not destroy the active principles, that aldehyde destroys both the oxidising itate and the reducing Schardinger, and that itate without aldehyde cannot oxidise the nitrite in the given time.

This dual action of acetaldehyde offers a possible explanation of an observation many times made that itate can in a given period oxidise a definite amount of sodium nitrite in the presence of acetaldehyde; but if the nitrite be presented in two portions, the sum of which is the same as the original amount, oxidation is never completed.

Exp. 8. Four tubes were half filled with 75 cc. milk, 1 cc. acetaldehyde, and 6, 7, 8 and 9 cc. standard sodium nitrite respectively. Rotated in thermostat at 45° and tested after one hour:

Table VIII.

Reagent	Amount of nitrite added			
	6	7	8	9
Griess-Ilosvay	-	-	-	-
Guaiacum	-	-	-	-
Diphenylamine	+	+	+	+

To the first three tubes there were then added 3, 2 and 1 cc. of sodium nitrite, *i.e.* sufficient to bring the total amount of nitrite to 9 cc. which amount was completely oxidised in one hour. Even after the lapse of $2\frac{1}{2}$ hours the tubes gave strong positive reactions for nitrite showing that the preliminary heating with aldehyde had destroyed the itate which should have been capable of oxidising the further added quantities of nitrite.

Similarly atite is destroyed by its accelerator acetaldehyde, a phenomenon illustrated in the following experiment.

Exp. 9. 150 cc. of milk with 2 cc. of 10 % aldehyde were rotated in the thermostat at 45° and periodically tested. After 15 minutes, the milk no longer gave a positive reaction with Guaiacum tincture and therefore presumably the itate was destroyed. The milk, which still contained acetaldehyde, was then divided into two equal portions, to one (A) were added 3 cc. of standard nitrite (strong) and to the other (B) 5 cc. of 4 % nitrate. A control (C) containing 75 cc. of milk, 1 cc. aldehyde and 3 cc. nitrite was also put up. The three tubes were rotated in the thermostat at 45° and periodically examined:

Table IX.

Time in minutes	Test	(A) Added nitrite	(B) Added nitrate	(C) Control
15	Griess	+	-	-
	Guaiacum	-	-	-
45	Griess	+	-	.
	Guaiacum	-	-	.
120	Griess	+	-	.
	Guaiacum	-	-	.

Since the control (C) by its negative reaction with Griess showed that the itate was active, the failure to destroy the added nitrite in (A) must be attributed to the disappearance of the itate, it having been inactivated by the preliminary treatment with aldehyde. Similarly the failure to produce nitrite in (B) must be due to the destruction of atite since, as is shown by (A), the preliminary heating with aldehyde had destroyed the itate. Otherwise expressed there are only two ways in which the absence of nitrite in (B) can be accounted for; one of these is the inactivation of atite and the other the excessive activity of itate in oxidising nitrite to nitrate; this latter alternative is however excluded by (A), wherefore it must be concluded that the atite had been destroyed.

It has been stated above that oxygen is essential for this destruction to take place; to show this the following experiment may be quoted.

Exp. 10. The oxygen was replaced by nitrogen in a mixture of 225 cc. milk and 3 cc. of acetaldehyde and rotated, under anaerobic conditions, in the thermostat at 45° . After $2\frac{1}{2}$ hours, 15 cc. of 4 % sodium nitrate were added, precautions being taken to exclude air, and the rotation continued. After the lapse of one hour the mixture was titrated for nitrite and gave the value 0.677; at the end of the second hour, the titration value was 1.42.

From this it follows that neither prolonged heating, nor the presence of aldehyde in the absence of oxygen impairs the activity of atite. After the titration was made the remainder of the milk was well aerated, replaced in the thermostat and tested with Griess-Ilosvay reagent after the lapse of 15 minutes. A negative reaction was obtained showing likewise that neither prolonged heating nor the presence of aldehyde in the absence of oxygen impairs the activity of itate.

By similar experimentation, details of which it is unnecessary to give, it was found that Schardinger reductase is destroyed by acetaldehyde in the presence of oxygen.

Summarising the evidence relating to the action of acetaldehyde; whilst acetaldehyde is an accelerator, it also in presence of oxygen destroys atite, Schardinger reductase, and itate, that is both reducing and oxidising systems. It is difficult to understand the function of the oxygen in the destruction of substances of so diametrically opposed properties.

IV. THE NATURE OF ATITE AND ITATE.

With respect to atite, Bach considers it to be an enzyme; Kastle and Elvove suggest it might be an oxidisable substance; and Harris and Creighton consider it to be an enzyme and term it reductase.

Any such views as may have been held with regard to atite by earlier authors do not apply to itate since this substance has not been previously described by other workers, although the fact of the disappearance of nitrite is mentioned by Bach. In deciding whether these substances are enzymes or oxidisable and reducible substances respectively, much depends on the relative importance attached to the characters of thermolability and of indestructibility in the normal reaction. For us the latter would appear the more important, and from the experimental evidence brought forward it seems that the most reasonable explanation of the nature of atite and itate is that they are respectively oxygen accepting and donating substances present in milk in limited amount; limited, in view of the fact that they are only capable of reducing or oxidising definite amounts of nitrate or nitrite respectively. At the same time we have not overlooked the possibility of the toxic effect of the aldehyde in gradually depressing the activity of both atite and itate in such a manner as ultimately to mask what to us appears the most characteristic feature of enzyme action, namely indestructibility in a normal reaction. The possibility of atite and itate being the oxidised and reduced forms respectively of one and the same substance, comparable with glutathione, is excluded for the reason that the amount of nitrite which can be oxidised by itate is very much greater than the amount of nitrite which the atite can produce in the course of a normal experiment.

SUMMARY.

1. Milk contains an oxidisable substance which has the power under certain conditions of reducing nitrate to nitrite. Although possessing one of the characteristics of enzymes, namely thermolability, the evidence indicates that it is not a true enzyme, in that it is destroyed in the process of reduction. It is precipitated by the methods commonly employed in coagulating milk. The name of *atite* is proposed for this substance pending more information regarding its nature.

2. Milk also contains a substance capable under certain conditions of effecting the oxidation of nitrite to nitrate. It is active only in the presence of oxygen and under the most favourable conditions will oxidise the nitrite formed by *atite* as quickly as it is formed, so that under the requisite conditions of the experiment the presence of neither of these two active bodies is discernible. This substance is termed *itate*, and the evidence points to the fact that it is not a true enzyme, in that it is destroyed in the course of its oxidative activity. Its characters resemble those of *atite* as regards thermolability and precipitation.

3. For both *atite* and *itate* an accelerator is required, aldehydes, especially acetaldehyde, being the most potent. The action of acetaldehyde is peculiar in that in the presence of oxygen it destroys *atite*, *itate*, and also Schardinger enzyme.

4. The reducing substance known as *atite* is distinct from the reducing enzyme of Schardinger.

5. Since the disappearance of the guaiacum-peroxide reaction of milk is coincident with the destruction of *itate* the latter is possibly identical with "peroxidase"; since moreover the conditions which bring about the inactivation of milk "peroxidase" do not similarly affect either the peroxidase of horse-radish or the oxidase of the potato it appears that milk peroxidase differs fundamentally in properties from plant peroxidases.

6. No direct physiological significance is attached to the presence of these two active principles, *atite* and *itate*; indeed it is difficult to rationalise such a circulation, nitrate \rightarrow nitrite \rightarrow nitrate, in a secretion. To what extent they are present in the milk of mammals generally has not been ascertained.

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LXXXV. STUDIES ON CARBOHYDRATE METABOLISM.

II. ON THE PREPARATION OF AN ANTI-DIABETIC HORMONE FROM YEAST. PART I.

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THE importance of phosphorus in the metabolism of carbohydrate is well known. Since insulin relieves the symptoms of diabetes, and carbohydrate-phosphorus compounds are formed by the action of yeast, it seemed possible that a substance similar to insulin might be obtained from this source. It has been shown [Winter and Smith, 1923, 1] that it is possible to extract from yeast a substance which has a similar effect to insulin in causing a lowering of the blood sugar of normal animals. Though this extract, like crude insulin, contains organic phosphorus, Dudley [1923] has shown that insulin purified by the picrate method contains no phosphorus, and that the phosphorus in the crude product has no significance in this connection. A beneficial effect was observed when the extract of yeast was injected into persons suffering from diabetes mellitus [Winter and Smith, 1923, 2]. Later Collip [1923, 1] obtained an anti-diabetic substance from a variety of vegetable sources, but he apparently found that yeast was not a satisfactory source, for he reported twenty failures before an active extract was obtained. Recently Funk and Corbitt [1923] have met with similar variation when yeast was used. We soon found that different samples of yeast gave extracts which differed widely in their activity, some being quite inactive.

In a private communication from Dr H. H. Dale we were informed that from experiments performed in his laboratory no confirmation of our results could be obtained; as much as 400 mg. of the extract was injected into a rabbit without any lowering of the blood sugar being obtained.

The following is a description of some experiments which we have undertaken to determine the conditions under which an anti-diabetic substance could be obtained from yeast. Collip [1923, 1] has suggested the name *gluco-kinin* for anti-diabetic hormones obtained from sources other than pancreas, and this name will be adopted in the following account.

EXPERIMENTAL.

The method employed for the extraction of the yeast was essentially that devised by Collip [1922] for the preparation of insulin from pancreas, and modified by Dudley [1923].

In our first experiments 2 kilos. of fresh baker's yeast were mixed with an equal volume of 95 % alcohol, and ground up with sand in a mortar. The extraction was allowed to continue at room temperature for two hours. The mixture was then filtered through a coarse pleated paper. Filtration was readily effected by this means. The remaining steps in the process are the same as in the pancreatic preparation, but unlike the extraction from pancreas, there is no difficulty caused by the separation of fat when the concentration of the alcoholic extract is nearly complete. Light petroleum was rarely necessary. The final product is a hygroscopic white powder readily soluble in water. For injection into animals a known weight of this was dissolved in normal saline. The injections were given subcutaneously, rabbits and rats being used as experimental animals. Food was withheld for 24 hours previous to injection. The blood sugar estimations were carried out by Bang's old method [1913].

The local reaction caused by injection of this extract was much less than in the case of insulin. This difference was particularly noticeable when cases of diabetes in man were being treated. The amount of extract necessary to lower the blood sugar to a similar level was much greater than that required when insulin was used. The effect of glucokinin would appear however to be more prolonged (see Fig. 1, p. 692). The action on the blood sugar may be seen from the following example.

Rabbit 2.2 kilos.					
Time					Blood sugar %
10.00	·14
10.10	injection of glucokinin ·06 mg./g. body weight				
11.30	·10
2.20	·06
4.30	·07
6.30	·09

Though the weight of extract from a given quantity of yeast varied, the same standard dose was always given to each animal (0.06 mg. per g. body weight).

When it was found that certain samples of yeast gave little activity it seemed possible that the properties of glucokinin might be slightly different from those of insulin, and that some modification in the alcoholic process might be necessary. Small samples of the same yeast were taken and extracted with different strengths of alcohol, and in the ensuing stages precipitation was carried out in 70 or 80 % alcohol. No appreciable difference in the activity was observed. The acidity derived from the yeast would appear to be sufficient to ensure the stability of the active principle, assuming that

its properties are similar to those of insulin, since no advantage was derived from the addition of acetate buffer, p_{H} 4.5, during concentration of the filtrate. Insulin is fairly stable in acid solution but is rapidly destroyed by alkali (ten minutes in $N/10$ sodium carbonate at 100°) [Dudley, 1923].

It seemed possible, however, that glucokinin might be present in the cells as a precursor from which it could be liberated by the action of alkali. Yeast which yielded very little active principle by the ordinary method was extracted with alcohol in alkaline solution, and acidified before concentration. No increase of activity in these cases was observed.

The final precipitation with 80 % alcohol causes a separation of a yellow oily liquid. A small loss occurs at this stage, some of the glucokinin being precipitated. The greater part, however, of the glucokinin remains in solution to be precipitated in 95 % alcohol at -7° .

The disintegration of the cells by grinding the yeast with sand in a mortar being obviously incomplete, the possibility of splitting the cells by rapid freezing and thawing was tested with a view to increasing the yield from samples of yeast deficient in glucokinin. Through the kindness of Mr W. B. Hardy, we were able to carry out the freezing at a temperature of -19° in the Low Temperature Station, Cambridge. The yeast was spread out in a thin layer on trays. When thoroughly frozen it was rapidly thawed under warm alcohol in a water bath. Little success resulted, and we were forced to conclude that some factor was lacking in the samples of inactive yeast, and that no more complete extraction would increase the yield of glucokinin. The following is an example.

Rabbit 2.1 kilos.					Rabbit 2.9 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.00	14	10.10	13
10.15	injected	(usual process)	...	12	10.30	injected with extract ob-	12
12.30	12	...	tained from frozen yeast	11
2.30	13	12.45	12
4.30		2.45	11
					4.40	12

Up to the present we had used only samples of baker's yeast obtained from the same source locally. Since it was possible that the freshness of the yeast might be of importance, samples of baker's yeast were obtained as fresh as possible, and immediately placed in alcohol. Variability was experienced as before, and no definite evidence could be brought to show that the freshness or otherwise of the yeast was responsible for this.

Experiments were next carried out to determine whether brewer's yeast might be more suitable as a source of glucokinin. Samples of yeast were skimmed from beers of different gravity, and placed as soon as possible under alcohol. Some samples were taken at the usual period (end of fermentation), others were collected when the fermentation was proceeding most rapidly. These were necessarily small as considerable quantities of the fermenting fluid had to be filtered before the necessary quantity of yeast could be collected.

Both top and bottom yeasts were also tested. The activity was usually poor, and in the case of a sample of bottom yeast the blood sugar rose. The following are some results obtained from brewer's yeast.

Rabbit 2.5 kilos.					Rabbit 2.3 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	11.00
10.30	injected with top yeast	extract of	of	...	11.15	extract of bottom yeast			...
12.20	2.00
2.30	5.00
5.00	7.00

Rabbit 1.6 kilos.

Time									Blood sugar %
10.00
10.15	extract of yeast	collected at height of fermentation							...
12.30
2.00
4.00

It was found that when the final precipitation was carried out (in 95 % alcohol at -7°) the greater part of the precipitate usually settled overnight. The remainder being finely divided took some days to settle. These two fractions were roughly separated. No variation in the activity occurred as will be seen from the following figures.

Rabbit 2.2 kilos.					Rabbit 2.3 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	10.45
10.30	first fraction injected			...	11.00	second fraction given			...
11.30	12.30
2.00	2.30
3.30	5.00

A comparison was next made between the amounts of glucokinase obtainable from samples of the same brand of commercial yeast obtained on different dates. These were treated in the same way, and gave such very divergent results as may be seen from the following figures.

Rabbit 2.7 kilos.					Rabbit 2 kilos.				
Time				Blood sugar %	Time				Blood sugar %
11.45	10.00
12.15	injected			...	10.30	injected			...
2.00	12.30
3.30	2.30
5.00	5.15
6.00					
7.00					
7.30	convulsions occurred								
7.45	40 cc. 10 % glucose injected								
8.15	appeared normal, eating								
9.00					

The batch which caused the convulsions in the above experiment was used in treating hospital cases.

It seemed possible that the activity of poor samples of yeast might be renewed by suitable treatment. It was also possible that a considerable

quantity of the active principle might reach the fermenting liquor as an exudate when the yeast was grown in a suitable medium. Fermentation experiments were carried out using glucose or fructose. Phosphate was added in some experiments in order to increase the rate of fermentation, for it was not impossible that the presence of glucokinin might be associated with the rapid metabolism of carbohydrate which was taking place. The reaction was stopped after different periods by the addition of alcohol. The mixture was then filtered, and the filtrate was made up to 80 % alcohol. The process was completed in the usual manner. Negative results were obtained in every case, confirming the experiment shown above when the yeast had been skimmed at the height of fermentation. We concluded therefore that fermentation was not necessarily associated with the production of increased amounts of glucokinin. Cultivation of the yeast was then attempted under conditions in which the growth and fermentation would be slower. It seemed possible that the presence of glucokinin might be associated with a certain nutritional state of the yeast cell, such as its content of glycogen. The unusual course was adopted of mixing the yeast with potatoes as a source of nutrition; it was hoped that by not supplying the yeast with preformed reducing sugar some other process possibly associated with the production of glucokinin might be brought into prominence. In case potatoes might also be a source of glucokinin, uncooked potatoes were extracted with alcohol and the usual white powder obtained. No effect was observed when this was injected into a normal rabbit. For the cultivation of the yeast the potatoes were boiled, mashed, cooled and mixed with water before the addition of the yeast. In these experiments as little water as possible was added in order to economise alcohol, but sufficient to make the mixture liquid.

Experiment. 500 g. of yeast were added to 500 g. of potatoes in 700 cc. water and well mixed. The mixture was placed in an incubator at 26° and allowed to remain 24 hours. A similar experiment was carried out with the addition of 20 g. di-sodium hydrogen phosphate. At the end of the period of incubation the action was stopped by the addition of alcohol and the process completed as before. The products obtained were tested by injection into rabbits with the following results.

Rabbit 3 kilos.					Rabbit 2.2 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	10.15
10.30	injected (+ phosphate)	10.45	injected (no phosphate)
12.30	12.45
2.30	2.40
3.45	4.00
5.30	6.00

The original yeast as received was almost inactive. The result of similar experiments has shown that the addition of phosphate increased the yield of glucokinin. A sample of French yeast was divided into two parts. The first

was treated by the ordinary method, the other was cultivated with potatoes in the presence of phosphate. The extracts had the following activity.

Rabbit 1.7 kilos.					Rabbit 1.9 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.00	10.15
10.15	injected	(+ phosphate)	10.30	injected	(original yeast)
11.00	12.00
12.20	2.00
12.30	convulsed	4.30
12.40					
1.00	40 cc. 10 % glucose	injected					
1.15	animal normal	and eating					
2.00					

The varying effect on different animals is shown in the following experiment in which the sample which convulsed the animal in the foregoing experiment was tested on another rabbit. Only a moderate fall in the blood sugar occurred.

Rabbit 2.1 kilos.					Blood sugar	
Time					%	
9.45	13	
10.00	injected		
11.30	10	
12.30	09	
3.00	10	
5.00	13	

These two experiments again emphasise the difference of effect which may occur when the same dose is given to animals of the same species, and this must always be a difficulty when tests of substances depend on their action on different animals.

In the above experiments very little grinding of the yeast was possible owing to the admixture with potatoes. It is probable that only very imperfect extraction of the cells by the alcohol took place, as it is well known that substances are extracted from cells only with great difficulty unless the cell membrane is thoroughly disintegrated. The active principle obtained in the above experiments may have been mainly derived from that which had already passed from the cells into the surrounding liquor during fermentation. Bacterial action cannot, of course, be excluded, but the fact that the temperature of incubation was 26° and that incubation was only continued for one day renders it likely that the glucokinase arose from the dominant organism, that is the yeast. Experiments were then carried out to determine whether rupture of the cell membrane would increase the yield of glucokinase. The yeast was mixed with potatoes as before, with and without phosphate. The mixture was then incubated at 26° for one day, and afterwards placed in the cold room at - 19° until thoroughly frozen. It was rapidly thawed under alcohol as before. No increase of activity resulted. The above series of experiments tended to show that the presence of glucokinase was not associated with ordinary growth or fermentation. Similar experiments in which the

mixture was maintained at different temperatures, such as 10° and 15°, gave negative results.

Yeast was allowed to autolyse at 45° for two days. The liquid was then treated in the usual manner. There was no evidence of the presence of glucokinase as a result of this variation. This was to be expected since insulin is destroyed by trypsin and pepsin [Dudley, 1923]. Although glucokinase may not be identical with insulin, it is reasonable to expect that its behaviour towards proteolytic enzymes would be similar. Some of the same yeast was dried in a current of warm air. It was then extracted with alcohol. The product obtained was without action on the blood sugar of a rabbit.

It was possible that rapid aeration might influence the formation of glucokinase. Yeast was mixed with an equal weight of potatoes to which phosphate was added. Enough water was then added to make the mass sufficiently liquid for aeration. A rapid current of air was drawn through the liquid overnight, the liquid being maintained at 26°. A sample of the same yeast was treated in a similar manner but without aeration. The results obtained from the two samples were as follows.

Rabbit 2.1 kilos.					Rabbit 1.8 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.20	10.30	13
10.45	injected	(without aeration)		14	11.00	injected	(with aeration)		13
12.30	10	12.45	13
2.00	10	2.15	12
4.30	13	4.45	13

Since the original yeast had a small content of glucokinase which was increased by incubation of the yeast with potatoes in the presence of phosphate, it might be concluded that the effect of aeration is to destroy any glucokinase which may originally be present. The destruction may be caused by oxidation, or it may be that the metabolism of yeast in the presence of excess of oxygen is altered, and carried out on different lines, which do not necessitate the formation of glucokinase. Since the precise conditions under which glucokinase is formed by the cells, and the effect of glucokinase on carbohydrate metabolism is unknown, it is impossible to come to any conclusion on this point at the present time. The result may also be explained on the basis that the responsible organism was not a yeast but some other type (possibly anaerobic) which was suppressed by aeration.

The foregoing experiments on the effect of fermentation, mixing with potatoes, temperature, and drying, were all made with yeast from the same distillery, which if extracted without additional treatment gave only small amounts of glucokinase. The results showed that the yield could not be increased to a marked extent: the only factor which appeared to have any influence was the presence or absence of phosphates, as seen in the cultivation experiments with potatoes. When phosphate was added the extract was sufficiently potent to cause convulsions in a rabbit. The similar treatment without phosphates gave no increase of activity. This fact merely serves to

emphasise the importance of phosphate in questions connected with carbohydrate metabolism, but the mechanism at work in the increased production of glucokinin is unknown. Harden and Young [1908] showed that the rate of alcoholic fermentation was increased by the addition of phosphate due to increased formation of hexosephosphoric acid being possible. Since rapid fermentation had no effect on the yield of glucokinin, the increased formation of hexosephosphate would appear to be unconnected with the formation of the active principle. It is possible that the effect of phosphates may not be the same on different yeasts as regards their glucokinin content, and that the effect may vary with other cultural conditions.

It seemed probable that samples of commercial yeast from different sources might vary in their content of glucokinin. Samples were obtained from different distilleries and tested. Further samples were obtained at intervals, and it was found that in most cases the yield of glucokinin was small, but that in one case there was constant activity, which however was not so great as that of the yeast used in our first experiments. It was possible that there might be some factor of nutrition which was only present in the process employed in this distillery, or that there might be present a small amount of a certain strain of yeast which was almost the sole origin of the glucokinin.

Experiments have been carried out on these lines, with the result that a strain of yeast has been isolated, which gives a yield of glucokinin greater than that ever obtained previously. This property seems to be maintained, as successive batches have continued to prove active.

An investigation into possible means of increasing the yield of glucokinin from such yeast, and on the factors which lead to its formation, is in progress. The results will be given in Part II of this paper.

DISCUSSION.

The fact that an extract of yeast causes a fall in the blood sugar of normal animals affords no proof that it contains an anti-diabetic hormone. In those experiments in which only a small fall of blood sugar occurred, it would be still more difficult to maintain that it was due to a hormone of this type, and not to the large amount of other substances which are also present in the extract, such as breakdown products of proteins. For this reason we have refrained from injecting large quantities of yeast extract, and from continuing the blood sugar determinations for longer than nine hours after the injection. The effect of starvation might possibly affect the condition of the animal to such a degree that the effect of the foreign substances, other than glucokinin, in the injection might begin to manifest themselves. We have emphasised the delayed action of glucokinin as compared with insulin, but this delay (as measured by the time when convulsions occur) we have never found to be more than eight hours.

In a recent paper Collip [1923, 2] gives some results of injecting an extract of yeast into normal animals. His results appear to be very different from those

which we have obtained. He uses several different processes (among them freezing) in obtaining his extract, but the action on the blood sugar appears to have been extremely delayed. No injection figures are given, but from the fact that one animal received nine injections in the course of twenty hours and another six in fourteen hours, it would appear that very large quantities of extract were administered. During the whole of this time the animal was without food. In the cases in which convulsions are recorded the animals died. It is necessary to emphasise here that in every case in which convulsions have occurred we have found complete recovery of the animal to ensue after injection of glucose. It would seem therefore that the anti-diabetic hormone which Collip claims was present in his extracts must be of a totally different type from the one present in our extracts, or that his extracts were so weak that vast quantities were necessary to obtain an effect. In this case the quantities of other substances present would probably have an injurious effect on the animal after more than a day's starvation with repeated withdrawals of blood. The Schaffer and Hartmann [1920] estimation of blood sugar was used by Collip. This method requires 1 cc. of blood for an estimation. We have always used Bang's old method, because of the small quantity of blood that is required, and the ease of obtaining it on an absorbent paper without serious inconvenience to the animal.

There appears to be little doubt that by using a suitable yeast a genuine anti-diabetic hormone can be obtained. It has been found [Winter and Smith, 1923, 2] that following administration of yeast extracts to diabetic patients, excretion of sugar is stopped, and the ratio copper reducing power to polarimetric value of the blood sugar is altered in the same way as after injections of insulin [Forrest, Smith and Winter, 1923].

MacLeod [1923] has suggested that such extracts may merely stimulate the secretion of insulin by the pancreas when injected into normal animals. The results with diabetic cases [Winter and Smith, 1923, 2] would appear to show that just as beneficial an effect may occur as when pancreatic insulin is used; and that therefore, in our opinion, the evidence is strongly in favour of a true anti-diabetic hormone being obtainable from suitable yeasts.

SUMMARY.

1. It is shown that a substance may be extracted from yeast which has an effect similar to that of insulin on the blood sugar of normal animals.
2. When convulsions occur as the result of the injection of this substance, the animals may be recovered by injections of glucose.
3. Samples of yeast obtained from different sources yield varying amounts of glucokinin.
4. By allowing the yeast to ferment under different conditions, it is found that only in the case of an originally active yeast is the glucokinin formation increased under certain cultural conditions.

We wish to express our grateful thanks to Professor F. G. Hopkins for his continued interest in this work, and for providing the large amount of material used in this necessarily expensive research.

Our thanks are due to Messrs D. and D. F. Scanlan for much help during the course of this work, to Dr H. H. Dale for keeping us informed of the progress of the work on insulin which has been carried on in his laboratory, to Messrs Bishop and Brooke for obtaining for us regular supplies of yeast from different sources, and to Messrs H. and G. Simonds, Reading, for giving us every help in the investigation of brewer's yeast.

One of us (W. S.) is indebted to the Scientific and Industrial Research Board, and one (L. B. W.) to the Medical Research Council for grants held during the course of this research.

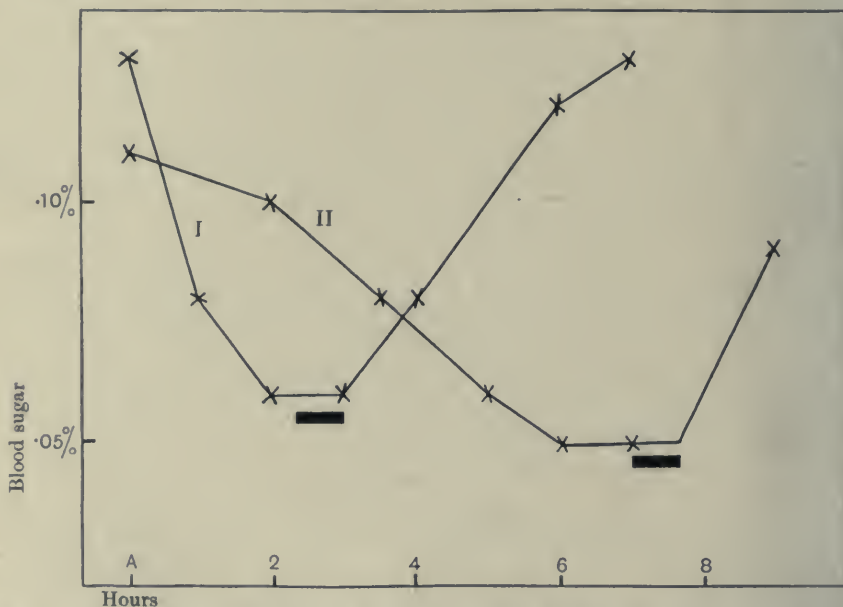


Fig. 1. Curves of blood sugar values of rabbits: I, after insulin (pancreatic); II, after yeast extract. At A the injection was given. The thick lines denote the period during which convulsions occurred. At the end of this period the animals were recovered by injections of glucose. The delayed action of the yeast extract is shown. Time in hours.

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LXXXVI. THE BASIC DISSOCIATION CONSTANT OF VALINE.

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(Received August 3rd, 1923.)

A KNOWLEDGE of the basic dissociation constant of valine was desired by the writer in connection with the methods which he has recently devised [1923] for estimating amino-acids by titrating under conditions governed by K_a and K_b values.

The present determination has been derived from the p_H -neutralisation curve. Known volumes of valine dissolved in water at a given concentration were treated with varying amounts of 0.1N HCl, and the resulting p_H values determined by means of the quinhydrone electrode. p_H was calculated from the observed E.M.F. reading by the formula

$$p_H = \frac{c - (\text{E.M.F. obs.})}{.059},$$

the value of the calomel half cell, c , being determined by reading against solutions of accurately known p_H : 0.1 HCl and buffer solutions of amino-acids plus HCl. All determinations were carried out in a box at 25°.

To obtain the "ideal titration curve," each reading was corrected for the amount of acid necessary to bring the solvent alone (without valine) to the same p_H and the same total volume, in the manner previously described by the writer [1923], and in accordance with the formula

$$i = d - \frac{g^e}{100}$$

where i = corrected amount of acid used in titrating to a given p_H ;

d = uncorrected amount of acid used in titrating to a given p_H ;

g = blank for 100 cc. (no. of cc. of N/10 acid necessary to bring solvent alone to the given p_H and to a final volume of 100 cc.);

e = final volume of titrated fluid, after addition of d cc. of HCl.

The value of g corresponding to each p_H reading is based on the formula

$$p_H = -\log \left(\alpha \cdot \frac{g}{0.001} \right)$$

and in practice is read from a curve constructed by the writer [1923].

In the table below are shown the results obtained in a typical titration of valine with HCl, and also the manner of introducing the blank correction to obtain the ideal curve.

Titration of 10 cc. of M/20 valine.

(d)	(e)	(f)	(g)	(h)	(i)
N/10 HCl added cc.	Total vol. of liquid after titration ($e = d + 10$) cc.	Corresponding p_H values	Blank for 100 cc. total vol. (read from curve) cc.	Blank for given total vol., e ($h = \frac{ge}{100}$) cc.	Corrected vol. of N/10 HCl added ($i = d - h$) cc.
0.1	10.1	3.98	—	—	0.1
0.4	10.4	3.38	0.5	0.05	0.35
1.0	11.0	2.93	1	0.1	0.9
1.3	11.3	2.79	2	0.2	1.1
1.6	11.6	2.69	2	0.2	1.4
2.0	12.0	2.58	3	0.4	1.6
2.3	12.3	2.51	4	0.5	1.8
3.0	13.0	2.34	5	0.7	2.3
3.2	13.2	2.29	5	0.7	2.5
3.4	13.4	2.24	6	0.8	2.6
4.0	14.0	2.12	8	1.1	2.9
5.0	15.0	1.96	12	1.8	3.2
6.0	16.0	1.85	15	2.4	3.6
7.0	17.0	1.78	19	3.2	3.8
8.0	18.0	1.69	23	4.1	3.9
9.0	19.0	1.64	25	4.8	4.2
10.0	20.0	1.58	29	5.8	4.2
12.0	22.0	1.51	35	7.7	4.3
15.0	25.0	1.46	40	10	5

Values in columns (g) are given to the nearest integer, in columns (h) and (i) to the first place.

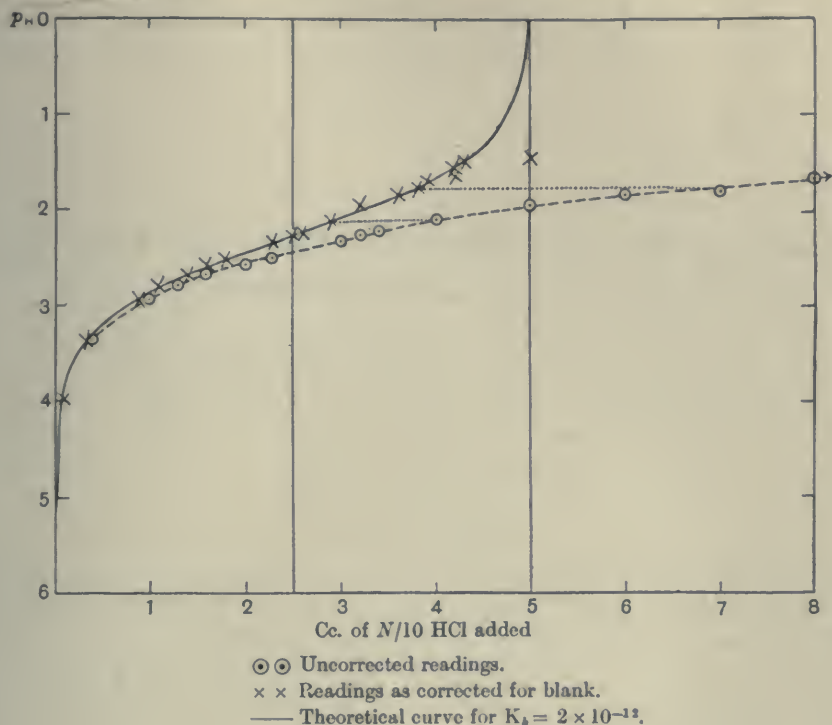
In successive titrations agreement between duplicates could be obtained to within about p_H 0.2.

In the diagram, circled points represent uncorrected readings, crosses the ideal corrected values deduced therefrom. The latter are in very close agreement with the titration curve¹ of a base with $K_b = 2 \times 10^{-12}$, which is represented by the unbroken line calculated from the formula

$$\log \frac{1}{[H^+]} = \log \frac{K_b}{K_w} + \log \frac{(1-a)}{a}.$$

The quinhydrone electrode has not previously been utilised for the determination of a dissociation constant, but it should prove of general applicability when used in conjunction with the above method. The writer has also found that provided it be properly standardised it may be conveniently employed for the accurate determination of amino-groups in amino-acids and generally of both basic and acidic groups.

¹ At the acid end of the titration the ideal corrected values tend to lie around the theoretical curve rather than to be strictly coincident with it, due to the very large magnitude of the blank corrections; a very slight percentage error in reading the correction, g , leading to a comparatively large error in the ideal corrected value.



SUMMARY.

Known amounts of HCl were added to valine and the resulting p_H values were determined by means of the quinhydrone electrode. Correcting for the effect of the solvent by the writer's method the ideal titration curve was deduced and showed a value of $14 - p_{K_b} = 2.3$, or $K_b = 2 \times 10^{-12}$ at a dilution of $\frac{M}{20}$, and $t = 25^\circ$.

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LXXXVII. ON THE SIGNIFICANCE OF THE ASH CONTENT OF CERTAIN MARINE ALGAE.

BY PAUL HAAS AND BARBARA RUSSELL-WELLS.

From the Botanical Department of University College.

(Received August 4th, 1923.)

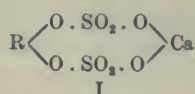
INTRODUCTORY.

COMPARATIVELY little attention has, in the past, been paid to the question whether certain ash constituents occurred as such in the plant or whether they had been produced during the process of incineration; thus while the sulphate or phosphate in an ash may be due to the accumulation within the plant of salts containing these radicles, they may, on the other hand, have been produced during incineration by the oxidation of organically combined sulphur or phosphorus, more especially if the ash has a strongly alkaline reaction, for in such cases the incineration amounts almost to alkali fusion.

It has long been known that many sea-weeds on incineration give ashes containing a relatively high percentage of calcium sulphate, but no particular significance was attached to this fact by earlier workers. By means of micro-chemical tests it should of course be easy to recognise the presence of certain ash constituents and so to establish their pre-existence within the plant before incineration. Such reactions, however, as can be carried out are due to dissolved ions and indicate the presence of salts which it should be possible to remove by dialysis. If such be the case no great significance attaches to their presence as they may merely be mobile products of metabolism or may be part of the plant's saline nutrients. If then a plant, or its aqueous extract still retains a considerable quantity of ash constituents even after exhaustive dialysis there is reason to suspect that such constituents are held in some form of chemical combination which precludes their free movement and consequently renders them incapable of dialysis.

It was found some time ago [Haas, 1921] that *Chondrus crispus* presents such a case, in which the ash is rich in calcium sulphate even after the material has been subjected to prolonged dialysis. Both chemical and micro-chemical analysis revealed the presence of ionised calcium but failed entirely to disclose any sulphate. To account for these facts it became necessary to

assume the existence in the plant of some form of masked sulphate such as an ethereal sulphate of the type represented by the formula



Chemical evidence for the correctness of this assumption was furnished by the fact that the masked sulphate could be converted into ionised sulphate by hydrolysis.

One of the consequences of this observation has been the recognition within the plant of colloidal electrolytes, a class of substances whose existence had not hitherto been suspected in this connection. The establishment of the presence of ionised calcium attached to a complex colloidal aggregate suggested the possibility for the manifestation of a variety of interesting physical chemical phenomena, of which osmotic pressure and conductivity are the most obvious. By means of the very simplest form of parchment osmometer it has been possible to demonstrate the existence of sufficient osmotic pressure to produce a steady rise in the stem of the thistle funnel. Conductivity measurements were kindly carried out for us by Mr F. C. Harwood in the physical chemistry laboratory of this college, and a preliminary account of some of his results has already appeared elsewhere [1923].

What may be the significance to the plant of the presence of osmotically active colloidal electrolytes in the cell wall is at present a matter of speculation. Whether these substances represent transitory phases in which the plant retains calcium sulphate in chemical combination, to be hydrolysed when occasion arises, it is not as yet possible to say; further evidence will have to be accumulated before it can be stated whether there is any seasonal variation in the occurrence of such compounds.

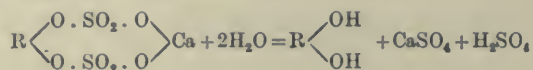
That the occurrence of ethereal sulphates is a fairly widely distributed phenomenon is shown by the present results in which the occurrence of similar compounds is described in the following Rhodophyceae and Phaeophyceae: *Ceramium rubrum*, *Delesseria sanguinea*, *Delesseria alata*, *Polysiphonia fastigiata*, *Plumaria elegans*, *Ascophyllum nodosum* and *Laminaria digitata*.

Since we commenced work on this subject Neuberg and Ohle [1921] have been able to establish by methods similar to our own the existence of the ethereal sulphate grouping in agar, the extract of a species of *Gelidium*. The physical chemistry of this substance has likewise since received attention at the hands of various workers, notably Samec and his collaborators [1921, 1922], and more recently Fairbrother and Mastin [1923].

In view of these facts it would appear necessary to urge the desirability of a more careful examination of the ash constituents of plant material in general with the object of elucidating, if possible, the nature of the combination of these substances in the plant.

METHODS.

In the case of *Chondrus* the proof of the existence of an ethereal sulphate depended on the fact that the percentage of sulphate contained in the hydrolysed solution of the hot extract (H.E.) was just twice that contained in the ash, in accordance with the equation:



from which it is clear that of the two molecular proportions of sulphate here given only the one combined with the calcium would be found in the ash, the other being lost during incineration.

In endeavouring to establish the existence of ethereal sulphate groupings in other material by similar means it has however not always been found possible to establish the exact relation of 2 : 1. The reasons for this discrepancy may be due either to experimental difficulties connected with the incineration or the hydrolysis, or to the inherent properties of the particular type of ethereal sulphate concerned.

A. With regard to difficulties encountered in the hydrolysis it has been found that the reaction is somewhat slowly effected by boiling with hydrochloric acid, due possibly to a tendency towards a reversal of the reaction as noted by Cunningham [1918]. The use of potassium chlorate or other oxidising agent during hydrolysis to assist in the destruction of the organic complex is of course precluded in this connection, since it is essential to ensure that all the sulphate found is due to hydrolysis and that none shall have been produced by the oxidation of organically combined sulphur. After a number of trials however the following conditions were found to effect the complete hydrolysis of such ethereal sulphates, the accuracy being confirmed by simultaneous estimation of total sulphur by Benedict's method.

About 0.75 g. of the substance is placed in a tall form beaker with 60 cc. of water to which 40 cc. of concentrated hydrochloric acid are added. The beaker is then covered with a clock-glass and heated over a sand-bath for six hours, water being added from time to time to replace that lost by evaporation. The resulting dark coloured solution is then filtered from any brown insoluble matter, which is nearly always present, and the filtrate is precipitated with barium chloride.

With regard to the incineration it has been found that the results obtained are very often rather more than half those obtained by hydrolysis. This is notably the case where the ash is strongly alkaline, e.g. *Laminaria* (p. 703), whereas on the other hand in the case of *Chondrus* hot extract, in which the ash is neutral, the ratio comes out exactly 2 : 1.

B. As regards the effect of the type of ethereal sulphate combination upon the ratio it must be borne in mind that the occurrence of an ammonium salt of the type $R \cdot O \cdot SO_2 \cdot ONH_4$ will at once throw out the ratio, since the

whole of its sulphate will be lost on incineration; such a case has already been described [Russell-Wells, 1922]. Similar remarks apply to the case of a true ethereal sulphate of the type $R_1 \cdot O \cdot SO_2 \cdot O \cdot R_2$ in which R_1 and R_2 are both organic complexes; in the case of such a compound of course the whole of the sulphate radicle should likewise be lost on incineration.

The general method of procedure may now be given, as it is essentially the same for all the cases investigated.

The carefully hand-picked material is extracted with hot water over a water-bath or in an autoclave at 105° ; the extract is then filtered over a Buchner funnel, first through calico and then through Chardin filter paper. The filtrate is thereupon placed in a parchment dialyser with a little thymol and the water is replaced morning and evening until it no longer gives any reaction for either sulphate or chloride; the dialysed liquid is evaporated to dryness on a platinum dish and the resulting scales are dried for analysis.

The necessity for thorough dialysis must be emphasised inasmuch as no great significance attaches to sulphate found in a hydrolysed solution unless precautions have been taken to ensure removal of any free sulphate, a consideration which is particularly important in the case of sea-weeds which are normally surrounded by a medium containing sulphates.

With the object of saving time a few analyses were carried out directly upon the weed, without preparing an extract, on the assumption that while adhering sulphate would undoubtedly throw out the ratio, indications of a distinctly higher proportion of sulphate on hydrolysis would be reliable evidence of the presence of ethereal sulphate. Although the method gave valuable indications in some cases it cannot be recommended owing largely to the fact that many weeds give a strongly alkaline ash, which tends to retain some of the sulphuric acid that should be lost on incineration; this is particularly the case with *Laminaria* (p. 703).

The weeds which have been examined for the presence of ethereal sulphate were all selected for their relatively high ash content and the high proportion of sulphate in their ash and it may be safely assumed that this is a reliable criterion for judging of the likelihood of such combinations occurring in any new plant material.

In deciding from the results of an analysis whether a substance contains ethereal sulphate, undue importance should not be attached to the establishment of the exact ratio 2 : 1; the deciding factor should rather be that the dialysed product yields on hydrolysis a relatively high proportion of sulphate and that this proportion exceeds that found in the ash.

Evidence of the occurrence of phosphoric acid in organic combination has also been obtained in the case of *Chondrus*, but here it is of course not possible to rely upon the difference in the amounts found on hydrolysis and incineration since phosphoric acid is not volatile; the evidence is therefore based entirely upon the establishment of phosphate in a dialysed solution after hydrolysis.

EXPERIMENTAL.

Chondrus crispus.

During the dialysis of the hot and cold extracts (H.E. and C.E.) of carrageen it was noticed that these substances, even when free from dialysable salts, drew a very considerable volume of water into the dialyser indicating that they must exert a definite osmotic pressure. To verify this, solutions of pure C.E. and H.E. of the same strength were placed in simple osmometers consisting of thistle funnels with pig's bladder stretched across them. In a short time a rise was observed in each case which was maintained for as long as the tube was left in position, a matter of about a week. A very much greater rise was observed in the case of the C.E. than in that of the H.E.

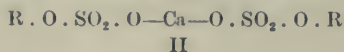
To obtain some measure of the osmotic pressure exerted by the C.E. in aqueous solution a freezing point determination was made with the following results:

A 1.5 % solution gave $\Delta = 0.045^\circ$ which corresponds to an osmotic pressure of 0.54 atmosphere.

Furthermore measurements kindly carried out for us by Mr F. C. Harwood showed the molecular conductivity at the same concentration to be 65.1 and that at infinite dilution to be 110, giving a value for the degree of ionisation of $\alpha = 0.59$.

A calculation of the minimum molecular weight of the C.E. from its calcium content of 4 % gives the value 1000, while from the depression of 0.045° the value 620 is obtained, which is in fairly close agreement with value 590 obtained by multiplying the theoretical molecular weight of 1000 by the degree of ionisation 0.59.

The evidence obtained from the conductivity measurements moreover points to a basicity of 2 for the acid radicle combined with the calcium, which lends support to the formula I (p. 697) previously ascribed to this substance in preference to the possible alternative formula II



With regard to the question of the occurrence of ethereal phosphate estimations were made on two occasions of the amount of phosphate in solutions of carefully dialysed H.E. when it was found that the amount contained in this material was 0.11 % calculated as P_2O_5 . We have however some hesitation in putting forward this figure as the correct value for the amount of phosphoric acid in the material, since we have experienced great difficulty in attempting to hydrolyse quantitatively samples of pure sodium glycerophosphate, a substance of relatively simple constitution when compared with H.E. Similar difficulty in hydrolysing ethereal phosphate has been experienced by Robison [1922].

Ceramium rubrum.

For this weed, as for all others, the ash content varies according to the amount of washing it has received previous to analysis, as may be seen from the following figures obtained from different samples: 11.58, 12.32, 13.05, 13.88, 25.41; it must however be borne in mind that excessive washing is to be avoided owing to the danger of losing some water-soluble constituents other than salts.

In the first instance a direct analysis of the weed was made without extraction; the sample selected was the fourth one quoted above which had been collected in the neighbourhood of Plymouth during the month of May. The following figures were obtained:

	%
Ash in the crude weed	13.88
SO ₄ in above ash	15.63
SO ₄ in weed by hydrolysis	4.13
SO ₄ „ „ incineration	2.17

It was then decided to examine an extract of the weed and for this purpose a sample obtained from Port Erin during the month of April was heated with water in an autoclave for one hour at 110°; after filtering, the residue was heated once more with a fresh quantity of water and the combined extracts were dialysed and evaporated; the dried material, weighing about 10 % of the air dry weed, forms clear light yellow gelatinous scales which swell up considerably in cold water and dissolve completely on standing without the application of heat.

Samples of the dialysed material from different sources gave the following figures on analysis:

	%	%	%
Ash in the extract	9.2	8.35	7.88
SO ₄ in the above ash	36.03	37.62	42.76
SO ₄ in the extract (by hydrolysis)	8.18	—	8.85
SO ₄ „ „ (by incineration)	3.31	3.14	3.37

It will be seen that in this case the ratios of the two sulphate estimations in the first and third cases are 2.47 : 1 and 2.62 : 1 respectively; as explained above this may be due either to the presence of ammonium groups or to the occurrence of a true organic ester; the matter is still under investigation.

To determine whether the substance has any osmotic properties a solution was prepared from 1.2 g. of the extract in 70 cc. of water. Placed in an osmometer there was a rise of 9.3 cm. in seven days thus showing that the substance exerts a distinct osmotic pressure.

Delesseria sanguinea.

In this case the weed, collected near Plymouth in May, was rapidly washed in two or three changes of water and then dried. Analysis of the air dry material gave the following figures:

	%
Ash in the washed weed	16.83
SO ₄ in above ash	44.15
SO ₄ in the weed (by hydrolysis)	14.47
SO ₄ „ „ (by incineration)	7.43

An extract of the above was prepared by heating for two hours in an autoclave at 110°; the mucilaginous solution on evaporation yielded clear gelatinous scales which are readily soluble in water. Analysis of the dialysed material gave the following results:

	%
Ash in the extract	16.01
SO ₄ in the above ash	68.39
SO ₄ in the extract (by hydrolysis)	23.84
SO ₄ " " (by incineration)	10.68

Osmotic properties were established by means of the osmometer in which a solution containing 1.5 g. of dialysed extract in 70 cc. of water registered a rise of 3 cm. in 40 hours. These facts together with the ratio of 2.23 : 1 for the two sulphates confirm the presence of ethereal sulphate.

Delesseria alata.

In this case only the weed itself was analysed. The material was kindly collected for us at Port Erin in July by Miss Knight, of Liverpool University. Analysis gave the following results:

	%
Ash in the washed weed	12.24
SO ₄ in the above ash	33.84
SO ₄ in the weed (by hydrolysis)	9.77
SO ₄ " " (by incineration)	4.17

Here again the excess of sulphate obtained by hydrolysis over that found on incineration proves the presence of ethereal sulphate.

Polysiphonia fastigiata.

The ash content of this weed again depends very much upon the amount of washing, as is shown by the following figures obtained from the same sample before and after washing:

	%	%
Ash before washing	24.88	28.19
Ash after washing	9.34	9.99

Analysis of an unwashed sample gave the following figures:

	%
Ash in the unwashed weed	24.88
SO ₄ " above ash	24.84
SO ₄ " unwashed weed (by hydrolysis)	11.80
SO ₄ " " " (by incineration)	6.18

An extract was made of a sample of weed kindly collected for us near Folkestone in the month of March by Mr Chater of this Department. Heated at 105° for one hour the weed yielded to water about 20 % of its weight, giving a mucilaginous solution which on evaporation left light brown shiny transparent scales.

The substance is somewhat sparingly soluble in cold water but dissolves readily on warming.

An analysis of this material after dialysis gave the following figures:

	%
Ash in the extract	13.95
SO ₄ „ above ash	61.89
SO ₄ „ extract (by hydrolysis)	21.65
SO ₄ „ „ (by incineration)	8.63

The remarkably high percentage of sulphate in the ash is noticeable and also the fact that the ratio of sulphates is 2.5 : 1. It is intended to investigate this matter further.

Plumaria elegans.

This weed, of which we have not seen any previously published analyses, is characterised by an ash containing a high proportion of silica as is shown by the following:

	%	%
Ash	17.5	17.09
Silica in ash	49.76	49.61

Extracted in an autoclave at 110° the weed yields about 10 % of its weight to water. The extract evaporated to dryness over a water-bath leaves dark very brittle scales which are only partially soluble again in water even on boiling; if however the extract is evaporated in a vacuum at room temperature the resulting scales behave quite differently in water, swelling up and going into solution on standing to form a mucilaginous liquid.

Analysis of the dialysed extract of a sample obtained from Port Erin in the summer gave the following:

	%
Ash in the extract	15.94
SO ₄ in the above ash	41.41
SO ₄ in the extract (by hydrolysis)	9.07
SO ₄ „ „ (by incineration)	6.6

Ascophyllum nodosum.

This weed was selected from among the Phaeophyceae on account of the high proportion of sulphate in its ash. The material here examined was kindly collected for us by Mr Chater near Folkestone during January. Extraction with water in an autoclave at 110° yielded a brown mucilaginous solution which was dialysed until free from adherent salts. On evaporating over a water-bath there remained opaque horny scales of a light chocolate brown colour which were insoluble in water; when however the liquid was evaporated in a vacuum at the temperature of the room the resulting solid was found to be completely soluble in cold water. The substance exhibits in a very marked degree the phenomenon of adhesion, and if dried on a glass or silica dish the scales in cracking off remove large flakes from the surface. The residual weed after exhaustive extraction with water in the autoclave still yields a quantity of substance to 5 % sodium carbonate from which it is precipitated on the addition of acid; this substance, which is presumably alginic acid, would appear to be distinct from the water-soluble

extract here described. An analysis of this substance gave the following figures:

	%
Ash in the extract	16.98
SO ₄ " above ash	55.34
SO ₄ " extract (by hydrolysis)	11.41
SO ₄ " " (by incineration)	9.40

The presence of ethereal sulphate may be deduced from these figures in spite of the fact that the ratio of the two sulphates is only 1.2 : 1, since the known high alkalinity of the ash of this weed readily accounts for the large amount of sulphate obtained on incineration.

Corroborative evidence for the existence of an ionised salt, *i.e.* of ethereal sulphate, was furnished by placing a 1.7 % solution of the dialysed material in an osmometer when a rise of 17.5 cm. was produced in three days.

Laminaria digitata.

The following analyses were obtained from a number of samples of the weed collected at different times and places:

	I	II	III	IV ¹
	%	%	%	%
Ash in the weed	32.36	48.99	13.53	13.9
SO ₄ " above ash	9.97	5.78	25.63	24.71
SO ₄ " weed (by hydrolysis)	3.12	2.11	2.69	2.15
SO ₄ " " (by incineration)	3.22	2.83	3.46	3.43

Attention may be drawn to the striking difference in ash content between the samples, due no doubt to a difference in the amount of preliminary washing; furthermore in consequence of the large amount of saline impurities, presumably other than sulphate, in samples I and II the percentage of sulphate calculated upon the ash is in these cases comparatively low.

It is worthy of note moreover that in all cases the percentage of sulphate on hydrolysis is actually rather less than that on incineration; nevertheless the presence of an appreciable amount on hydrolysis suggests the possibility of ethereal sulphate. It seemed probable therefore that the ratio might have been thrown out by the incineration sulphate being too high, owing to the alkalinity of the ash. That this explanation is probably correct was shown by a comparison of the alkalinity of the ash of the weed with that of the ash of the extract. For this purpose samples of both weed and extract were incinerated and aqueous extracts of equal weights of the two ashes were titrated against *N*/50 hydrochloric acid with the following results:

	<i>N</i> /50 acid required
Alkali from 0.0388 g. of ash of weed	5.85 cc.
" " " " extract	0.24 cc.

Further confirmation was obtained by the analysis of a dialysed autoclave extract of the weed whose analysis is quoted above under column IV. This sample gave the following results:

	%
Ash in the extract	17.67
SO ₄ " above ash	50.19
SO ₄ " extract (by hydrolysis)	15.17
SO ₄ " " (by incineration)	8.87

¹ This sample was kindly collected for us at Menai Bridge in May by Miss Purvis of University College, Bangor.

It will be seen how different a result is obtained from an analysis of the weed itself and from an extract thereof; the ratio of the two sulphates is now 1.71 : 1 owing no doubt mainly to the lower alkalinity of the ash permitting a much larger proportion of the sulphate to escape on incineration; furthermore the removal of the extraneous salts by dialysis has doubled the percentage of sulphate in the ash as compared with that of the weed. The presence of ethereal sulphate is therefore established in the water-soluble extract of *Laminaria*.

Cetraria islandica.

This lichen was selected for comparison with the above mentioned algae not because it was suspected of containing ethereal sulphate but owing to the general similarity in the physical properties of its aqueous extract with those obtained from these algae.

The following figures were obtained by analysis of a commercial sample of "Iceland Moss"; the material was air dried and not washed.

	%
Ash in the crude lichen	3.58
SO ₄ " above ash	4.53
SO ₄ " lichen (by hydrolysis)	0.02
SO ₄ " " (by incineration)	0.16

From the above figures it is clear that this material contains no ethereal sulphate grouping and incidentally it is shown that the gelatinising properties of the substance are in no way dependent upon the existence of such a grouping.

In order to ascertain whether the mucilaginous substance extracted from Iceland moss exerted any osmotic pressure an extract was prepared and dialysed; it was then evaporated and a 1.5 % solution was placed in an osmometer but no rise whatever was recorded after three days.

SUMMARY.

1. From the evidence furnished it appears that there exist among the marine algae a number in which calcium, and probably sodium and potassium, sulphate occur in chemical combination with an organic complex in the form of an ethereal sulphate.

2. The presence of such ethereal sulphates is established by a comparison between the amounts of sulphate contained in the ash and in the hydrolysed solution of the material concerned.

3. For the purpose of such a comparison it is best to employ a dialysed aqueous extract, although in some cases conclusive evidence is furnished by an analysis of the weed itself.

4. The ethereal sulphates above referred to have their metallic constituent freely ionised but the sulphate is masked by being in combination with a complex colloidal aggregate; they accordingly belong to the group of colloidal

electrolytes and as such have a measurable conductivity and exhibit osmotic phenomena.

5. Attention is drawn to the occurrence in the cell wall of a mucilaginous material having osmotic properties, and the question of its significance to the plant is thereby raised.

In conclusion the authors wish to express their indebtedness to the Directors of the Marine Biological Stations at Plymouth and Port Erin for kindly obtaining for them supplies of much of the material required for this investigation.

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LXXXVIII. THE INFLUENCE OF COD-LIVER OIL, LINSEED OIL AND OLIVE OIL ON THE ASSIMILATION OF CALCIUM AND PHOSPHORUS IN THE GROWING PIG.

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THE literature of rickets contains many references to the use of fats and oils, especially cod-liver oil, as therapeutic agents. Gobley [1844] reported the successful use of ray's liver oil. Roloff [1866] advocated the use of oil-rich foods for the prevention of osteomalacia in farm animals. Kassowitz [1884, 1913] used cod-liver oil, and also other oils, as a vehicle for the administration of phosphorus. He did not however consider cod-liver oil of any special value *per se*, and notes that, in some cases, the administration of the oil alone had no beneficial action in curing rickets.

Schabad [1909], Birk [1909], Meyer [1913] and others have found that the administration of phosphorus in cod-liver oil increased the retention of calcium and phosphorus. On the other hand, Flieger [1897] showed that, in some cases at least, the phosphorised oil was ineffectual as a cure for rickets, and Schabad and Sorochowitsch [1911] and Schloss [1914] found that its use was not always followed by an increased retention of calcium and phosphorus.

Schloss, and Schabad and Sorochowitsch both found that an increased retention of calcium and phosphorus followed the administration of calcium salts in cod-liver oil.

The influence of fat without the addition of minerals has been studied by several workers. Kochmann and Petzsch [1911] found that the addition of fat to the diet reduced the absorption of calcium. On the other hand, Herter [1898], working with pigs, noted that, on the reduction of fat in the diet, the absorption of phosphorus from the intestine was decreased.

Orgler [1912] found that cod-liver oil increased the retention of calcium. Steinitz [1903], Rothberg [1907], Meyer [1908] and Orgler noted a decreased retention of calcium on increasing the amount of milk fat in the diet. Telfer [1921] investigated this apparent difference in the action of butter fat and cod-liver oil. In his experiments, no difference in calcium retention occurred with variations in the nature of the fat.

The recent literature on the therapeutic value of cod-liver oil in rickets need not be referred to. The work has been of a pathological, rather than a physiological nature. Interest has centred chiefly in bone lesions, and results have been judged chiefly by skiagraphic or histological examinations.

At this Institute during the past three years a series of investigations has been carried out on the mineral requirements of the growing pig, and on certain factors which affect the assimilation of the minerals. One of these investigations seemed to indicate that, in the pig at least, an important factor in the production of rickets is a lack of correspondence between the mineral content of the diet and the mineral requirements of the animal [Elliot, Crichton and Orr, 1922]. But it was found that the addition of cod-liver oil to a ration deficient in calcium led to an immediate increase in the amount of both calcium and phosphorus absorbed and retained [Orr and Husband, 1922].

In view of this finding and of the contradictory results of the previous workers referred to above, it seemed desirable to investigate further the influence of oils on the assimilation of calcium and phosphorus. It was intended to determine the relative influence of cod-liver oil, linseed oil and olive oil on the absorption and retention of these minerals. It was thought also that the results would show the influence of oil *per se*, and also whether the vitamin-rich cod-liver oil had a specific action.

EXPERIMENTAL METHODS.

The experiments were carried out with young hog pigs two to four months old. The animals used in each experiment were taken from the same litter and were as comparable in weight as possible. They were confined in metabolism cages in which it was possible to collect urine and faeces separately. The urine was collected in 24 hour periods. The faeces were collected as soon as possible after excretion, and placed in weighed tins with tight-fitting lids. At the end of the 24 hour period the tins were again weighed, the contents thoroughly mixed, and duplicate samples taken for immediate analysis. The cages were washed out daily with distilled water, and the washings added to the urine.

To get the pigs accustomed to the metabolic cages, to the diet, and to the routine of the experiment, they were confined in the cages for about a week before the analysis of the urine and faeces began. During this period they were fed to the limit of their appetites. The amount of food fed during the experimental period was regulated according to the amount eaten during this preliminary period, and was kept constant throughout the experimental period.

Basal Diet. The basal diet used in all experiments consisted of maize meal, middlings (wheat offal), oatmeal and blood meal in the proportions stated below. Before each experiment, samples of the food-stuffs were taken from

bulk, and the percentages of N, CaO and P_2O_5 determined. The following figures are the averages for all the samples. The exact intake of N, CaO and P_2O_5 in each experiment is stated in the tables giving the results of the experiments.

Basal diet.

	Proportions	Average composition %		
		Total N	CaO	P_2O_5
Maize	10	1.48	0.008	0.633
Middlings	10	2.72	0.082	1.615
Oatmeal	10	2.15	0.077	1.084
Blood meal	1	12.96	0.226	0.380

The calcium content of this ration is very low, containing only 0.061 % of CaO, whereas the dry matter of sow's milk contains 2.37 % CaO.

Golding, Zilva, Drummond and Coward [1922] state that they had difficulty from a technical point of view to reduce the calcium intake of pigs below 0.338 % (0.47 % CaO). From their data it would appear that their sample of wheat offal contained an abnormally high percentage of calcium. The highest figure obtained in our analyses for wheat offal was 0.12 % CaO with which the figures given by Forbes [1913] and König agree.

The food was made into a thick paste with distilled water, and the animals had distilled water *ad lib.* to drink.

As it appeared from previous experiments that the influence of the oils would be found to be greatest when the mineral content of the ration was ill-balanced, and especially when there was a deficiency of calcium, it was decided not to adjust completely the mineral matter of the ration in the first series of experiments. The results of previous work however had shown that, with such a low supply of calcium, as was present in the diet, there was a danger of pathological symptoms developing during the course of the experiment. The amount of calcium was therefore increased by the addition of 50 cc. of a 20 % solution of $CaCl_2$, which is equivalent to about 2.6 g. of CaO. This raised the CaO content of the ration to between 3 and 4 g., which is not more than half of what has been found in this Institute to be the requirement of a rapidly growing young pig three or four months old.

In Exp. 3 a further adjustment of the mineral matter was made, to determine whether the effect of the oil would still be apparent with an ample supply of calcium in the food. Details of the adjustment are given with the data for the experiment. Though the excreta of each 24 hour period in Exps. 1 and 2, and of each 48 hour period in Exp. 3, were analysed separately, the averages for longer periods are given in the tables to economise space. The lengths of the periods for which the averages are given are not identical in every case. This variation is due to the fact that there was sometimes a delay of a day in introducing or withdrawing the oil if the faecal excretion of the preceding day had not been normal in amount. Even with fairly long experimental periods, it is desirable to keep in view the danger of getting a

fallacious result owing to an abnormally large or small expulsion of faeces in the 24 hours.

The following methods of analysis were used: *total nitrogen* by the Kjeldahl method; *calcium* by precipitation as oxalate and titration with $N/10$ $KMnO_4$; *phosphorus* by the Pemberton-Neumann method.

EXPERIMENTAL DATA.

Three experiments were conducted. In each experiment one animal received cod-liver oil, one linseed oil and one olive oil.

In the first experiment the comparative effect of the three oils was tested.

This experiment was divided into three periods, the "pre-period" when the basal ration only was fed, the "oil period" when the oil was added, and the "post-period" when again only the basal ration was fed. The amount of oil added was in each case 40 cc. per day.

Experiment 1.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms	
			At beginning of experiment	At end of experiment
28	Cod-liver oil	17 weeks	26.9	43.5
24	Linseed oil	17 "	26.6	44.9
26	Olive oil	17 "	24.2	38.1

Ration for Nos. 28 and 24 was 1550 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

Ration for No. 26 was 1240 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

It will be seen from a study of Table I that the calcium balance of all three pigs showed a progressive increase throughout the experiment.

In Exp. 2 the pre-oil period was lengthened to ascertain what course the excretion of calcium and phosphorus would follow on the basal ration alone.

Experiment 2.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms	
			At beginning of experiment	At end of experiment
67	Cod-liver oil (40 cc.)	12 weeks	20.1	28.3
68	Linseed oil (30 cc.)	12 "	19.4	28.3
70	Olive oil (30 cc.)	12 "	17.3	29.9

The ration for Nos. 68 and 70 was 930 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

The ration for No. 67 was 1240 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

In this experiment it will be noted that all three pigs showed a negative calcium balance at the end of 19 days. At this period it was decided to add

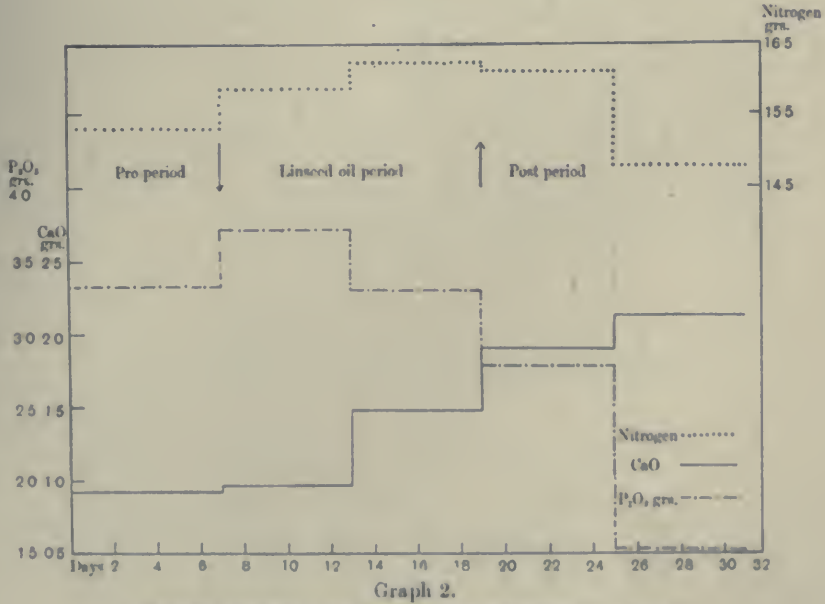
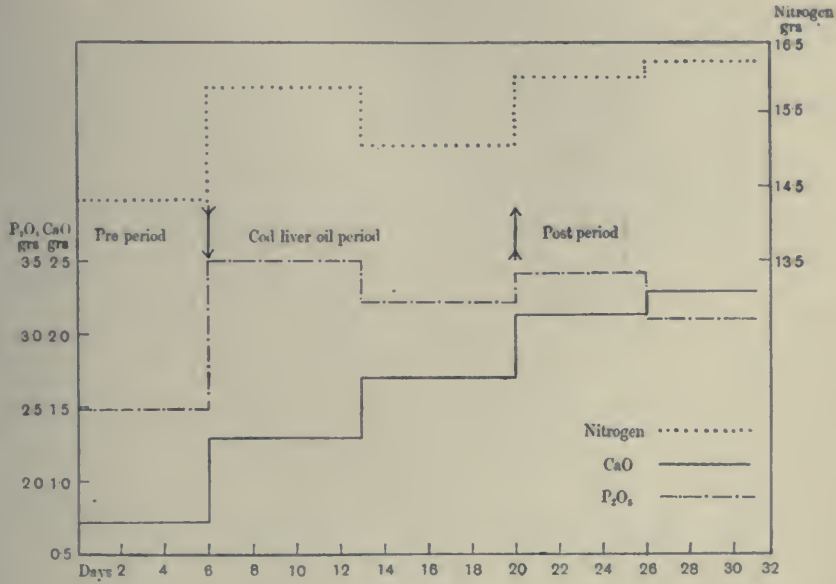


Table I. *Average daily excretions and balances in grams.*

Pig No. 28. Cod-liver oil. Duration of experiment: 31 days.

Average daily weight of faeces			Nitrogen				CaO				P ₂ O ₅			
			Intake		Balance		Intake		Balance		Intake		Balance	
			Urine	Faeces	Total	Urine	Urine	Faeces	Total	Urine	Urine	Faeces	Total	Balance
Pre-period Oil period	6 days	919	38.70	14.54	9.85	24.39	14.31	3.54	0.32	2.50	18.07	5.30	10.29	2.48
	1st 7	757	"	14.56	8.29	22.85	15.85	"	0.21	2.04	"	5.58	9.00	3.49
	2nd 7	891	"	13.35	10.28	23.63	15.07	"	0.14	1.70	"	5.03	9.83	3.21
	Post-period 1st 6	803	"	13.12	9.59	22.71	15.99	"	0.12	1.30	"	4.87	9.79	3.41
	last 5	792	"	13.36	9.15	22.51	16.19	"	0.12	1.14	"	4.89	10.08	3.10
Pig No. 24. Linseed oil. Duration of experiment: 31 days.														
Pre-period Oil period	7 days	883	38.70	12.89	10.51	23.40	15.30	3.54	0.21	2.40	18.07	4.99	9.75	3.33
	1st 6	845	"	13.30	9.54	22.84	15.86	"	0.15	2.43	"	4.88	9.48	3.71
	2nd 6	872	"	13.40	9.10	22.50	16.20	"	0.12	1.95	"	5.04	9.73	3.30
	Post-period 1st 6	869	"	13.30	9.31	22.61	16.09	"	0.11	1.53	"	4.89	10.40	2.78
	last 6	889	"	14.68	9.23	23.91	14.79	"	0.12	1.29	"	4.77	11.78	1.52
Pig No. 26. Olive oil. Duration of experiment: 28 days.														
Pre-period Oil period	2 days	732	30.96	10.41	8.10	18.51	12.45	3.36	0.18	2.26	14.46	3.96	8.04	2.46
	1st 7	666	"	11.47	7.32	18.79	12.17	"	0.18	2.04	"	4.21	8.10	2.15
	2nd 7	661	"	11.00	7.68	18.68	12.28	"	0.15	1.71	"	3.88	7.45	3.13
	Post-period 1st 6	663	"	10.92	7.68	18.60	12.36	"	0.12	1.58	"	4.03	7.99	2.44
	last 6	611	"	11.16	6.98	18.14	12.82	"	0.12	1.42	"	4.06	8.44	1.96

NOTE:—At the commencement of the experiment pig No. 26 received the same ration as pigs Nos. 28 and 24. Unfortunately on the first day of the collection for analyses the pig refused to consume its whole ration. The ration was therefore reduced. In the analytical data for this pig only the last two days' analyses of the pre-period were taken.

Table II. *Average daily excretions and balances in grams.*

Fig No. 70. Linseed oil. Duration of experiment: 40 days.

Days	Average daily weight of faeces	Nitrogen			CaO			P ₂ O ₅		
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total
1-5 inclusive	532	22.66	8.63	6.07	15.30	7.36	3.30	0.19	2.07	2.26
6-12 "	524	"	9.14	6.59	15.73	6.93	"	0.12	2.47	2.59
13-19 "	478	"	9.88	5.74	15.62	7.04	"	0.10	2.65	2.75
20-26 "	467	"	9.86	5.33	15.19	7.47	"	0.10	3.26	3.36
27-33 "	481	"	9.82	5.05	14.87	7.79	"	0.12	3.27	3.39
34-40 "	467	22.89	9.21	4.59	13.80	9.09	3.13	0.11	3.06	3.17
										-0.04
							9.44	1.09	7.06	8.15
										1.29

Fig No. 67. Cod-liver oil. Duration of experiment: 40 days.

Days	Average daily weight of faeces	Nitrogen			CaO			P ₂ O ₅		
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total
1-7 inclusive	638	30.21	10.35	8.52	18.87	11.34	3.48	0.12	2.72	2.84
8-13 "	621	"	11.00	7.47	18.47	11.74	"	0.13	3.18	3.31
14-19 "	716	"	11.48	7.89	19.37	10.84	"	0.15	3.69	3.84
20-22 "	726	"	11.81	8.03	19.84	10.37	"	0.19	3.77	3.96
23-28 "	446	20.14	9.49	5.34	14.83	5.31	2.32	0.12	2.03	2.15
29-34 "	347	"	8.43	4.72	13.15	6.99	"	0.07	0.71	0.78
35-40 "	237	15.25	7.35	3.25	10.60	4.65	1.70	0.05	0.49	0.54
										+1.16
							12.59	2.42	6.86	9.28
							"	1.99	7.59	9.58
							"	1.93	9.06	10.99
							"	1.73	9.36	11.09
							8.40	2.45	5.39	7.84
							"	2.29	3.58	5.87
							6.30	2.12	2.63	4.75
										1.55

Fig No. 68. Olive oil. Duration of experiment: 38 days.

Days	Average daily weight of faeces	Nitrogen			CaO			P ₂ O ₅		
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total
1-4 inclusive	470	22.65	9.07	6.24	15.31	7.35	3.30	0.14	2.11	2.25
5-10 "	429	"	9.30	5.55	14.85	7.81	"	0.12	2.17	2.29
11-16 "	474	"	9.77	5.74	15.51	7.15	"	0.12	3.10	3.22
17-20 "	432	"	9.80	5.29	15.09	7.57	"	0.13	3.28	3.41
21-26 "	454	"	10.14	5.74	15.88	6.78	"	0.15	3.29	3.44
27-32 "	401	"	10.17	5.12	15.29	7.37	"	0.15	2.36	2.51
33-38 "	437	22.98	10.15	5.33	15.48	7.50	3.07	0.14	2.24	2.38
										+0.69
							9.45	1.89	5.15	7.04
							"	2.08	5.03	7.11
							"	1.84	6.20	8.04
							"	1.50	6.41	7.91
							"	1.91	6.54	8.45
							"	2.17	5.33	7.50
							9.43	2.19	5.47	7.66
										1.77

NOTE. —————> indicates the introduction of the oil.

Table III. *Average daily excretions and balances in grams.*

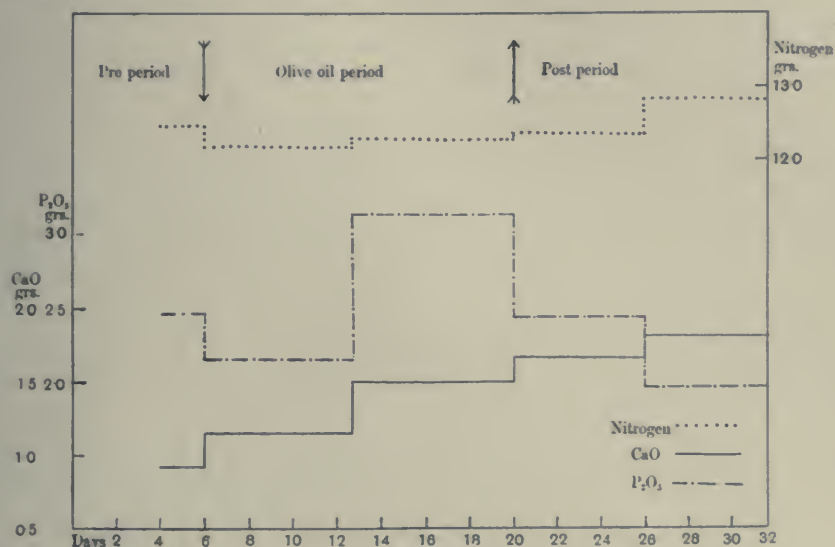
		Pig No. 669. Cod-liver oil. Duration of experiment: 20 days.									
		Nitrogen				CaO				P ₂ O ₅	
		Intake	Urine	Faeces	Total	Intake	Urine	Faeces	Total	Intake	Urine
		Average daily weight of faeces			Balance				Balance		
Days	1-6 inclusive	490	22.77	9.56	5.91	15.47	7.30	3.54	0.11	2.56	2.67
"	7-10 "	467	"	8.87	5.78	14.65	8.12	"	0.15	2.91	3.06
"	11-14 "	459	22.88	10.75	5.55	16.30	6.58	3.51	0.12	3.74	3.86
"	15-18 "	517	22.98	10.12	6.07	16.19	6.79	3.49	0.08	2.50	2.58
"	19-20 "	643	"	11.02	6.50	17.52	5.46	"	0.11	1.79	1.90
Pig No. 670. Olive oil. Duration of experiment: 40 days.											
Days	1-6 inclusive	515	22.77	10.25	6.52	16.77	6.00	14.59	0.04	11.92	12.56
"	7-12*	512	22.98	10.93	6.33	17.26	5.72	14.55	0.61	13.03	13.61
"	13-18 "	510	"	11.19	5.96	17.15	5.83	14.52	0.50	13.27	13.77
"	19-22 "	557	"	9.74	5.83	15.57	7.41	"	0.36	14.17	14.53
"	23-28 "	540	"	11.03	5.42	16.45	6.53	"	0.45	13.68	14.13
"	29-34 "	503	"	10.63	5.21	15.84	7.14	"	0.39	12.79	13.18
"	35-40 "	471	"	10.13	5.11	15.24	7.74	"	0.44	12.68	13.12
Pig No. 671. Linseed oil. Duration of experiment: 42 days.											
Days	1-4 inclusive	480	22.78	11.84	6.30	18.14	4.64	3.54	0.12	3.17	3.29
"	5-10 "	458	"	10.82	5.47	16.29	6.49	"	0.13	3.07	3.20
"	11-16 "	445	22.84	10.88	5.23	16.11	6.73	3.52	0.16	3.60	3.76
"	17-20 "	428	22.98	10.80	5.08	15.88	7.10	3.49	0.17	3.82	3.99
"	21-26 "	446	"	10.26	5.10	15.36	7.62	"	0.15	3.88	4.03
"	27-32 "	480	"	10.26	5.36	15.62	7.36	"	0.14	3.74	3.88
"	33-38 "	474	"	10.68	5.01	15.69	7.29	"	0.16	3.63	3.79
"	39-42 "	454	"	10.63	4.79	15.42	7.56	"	0.15	3.54	3.69

→ indicates the introduction of the oil.

* Ca₃(PO₄)₃ introduced on this day.

† Average for four days only, figures for the first two days after the addition of Ca₃(PO₄)₃ being rejected.

10 cc. of cod-liver oil to the ration of pig No. 67 and 30 cc. of olive oil to the ration of pig No. 68. Pig. No. 70 was continued on the basal ration alone for a further period of 14 days as a control, to ascertain whether the negative calcium balance would continue.



Graph 3.
Average balances for Pig No. 26. Experiment 1. Table I.

At the end of this time 30 cc. of linseed oil were added to the ration of this pig. It will be seen from Table II that the addition of the oil to the ration resulted in a rapid conversion of the negative calcium balances to positive balances in the case of pigs Nos. 67 and 68, and in a lowering of the negative balance of pig No. 70.

Experiment 3.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms	
			At beginning of experiment	At end of experiment
669	Cod-liver oil (30 cc.)	14 weeks	19.7	25.8
670	Olive oil (30 cc.)	14 "	20.6	31.0
671	Linseed oil (30 cc.)	14 "	25.1	35.5

Ration for Nos. 669 and 671 was 930 g. of basal ration plus 50 cc. of 20 % CaCl_2 solution.

Ration for No. 670 was 930 g. of basal ration plus 26 g. of chalk and 5 g. of NaCl.

In Exps. 1 and 2 the mineral matter of the diet was ill-balanced, especially with regard to calcium, sodium and chlorine, which were markedly deficient. In this experiment those deficiencies were made good in the case of one of the pigs, No. 670, by the addition to the day's ration of 26 g. of CaCO_3 , and 5 g. of NaCl. On the 12th day of the experiment 8 g. of tricalcium phosphate

were substituted for 6 g. of the CaCO_3 , to adjust the phosphorus content of the ration. This ration thus adjusted contained the mineral ingredients in amounts approximately equal to those contained in an amount of sow's milk of equal caloric value. Of course, though these various minerals were present roughly in the same proportion as those found in sow's milk, it must not be assumed that they were present in the same utilisable form.

DISCUSSION OF RESULTS.

It will be seen from Tables II and III that prior to the addition of oil there was a progressively increasing excretion of both calcium and phosphorus. This occurred in every pig used in these two experiments. In every case where the pre-period was prolonged, the calcium balance became negative, and the phosphorus balance correspondingly reduced.

The increased excretion is in the faeces. The urinary excretion of both calcium and phosphorus in pig No. 70 decreased in the pre-period. This happened also in pigs 68 and 670.

All the pigs were in the metabolism cages for a period of not less than seven days before analysis of the excreta was begun. It is most probable that during this preliminary period also, there was a decrease in the assimilation and retention of both calcium and phosphorus. The amounts retained even at the beginning of analysis were small compared with the normal requirement for growing pigs, which has been found here to be about 4-6 g. per day for pigs of the age used in the experiments.

Correlated with the decreased retention of calcium and phosphorus, a loss of appetite was noted. As the experiments proceeded, it became difficult to get the pigs to consume the daily ration. Pig No. 67 (Exp. 2), shortly after the introduction of cod-liver oil into the ration, could not be induced to consume the full ration, and similarly pig No. 669 (Exp. 3) finally refused its food, and work with it had to be abandoned.

A notable exception to this loss of appetite was pig No. 670, which had the adjusted mineral matter. Although the calcium balances showed the same downward course as with the other pigs, it was always eager for its food. Shortly after the experiment began its appetite improved and it would have eaten much more than the ration which satisfied it in the preliminary period.

It is interesting to note that this was the only pig used in these two experiments, which at the end of the experiment was in good condition. It was still active and apparently in good health. All the other five were beginning to show signs of ill health.

There is not sufficient data available for a profitable discussion of the cause of this decreased assimilation and retention of calcium and phosphorus.

The point of importance in the present investigation is that the diet on which the oils were superimposed was a diet on which normal assimilation and retention of calcium and phosphorus did not occur. The close confinement

in the metabolism cages was very probably a contributing factor, but the fact that, under the same conditions and with the same animals, assimilation and retention markedly increased on the introduction of oil to the diet demonstrates quite clearly the beneficial effect of oil on the metabolism of these minerals.

Oil period and post-oil period. The introduction of the oil, whether cod-liver, linseed or olive oil, is followed in all cases by a definite increase in the retention of calcium and phosphorus. In Exp. 1 where the pre-period was too short for the animals to reach a negative calcium balance, the calcium retention within seven days exceeded that of the pre-period. In general, the effect on calcium is more marked than on phosphorus. There is a correlation between calcium and phosphorus retention in growing animals, as has been noted by Fraps [1918] and others. This has been observed in all the metabolic experiments with growing pigs done in this Institute.

It has been noted above that, in the pre-period, the increased excretion of calcium and phosphorus that occurred when the pigs were continued on the basal ration, was due to increased elimination in the faeces. The increased retention on administration of the oil is brought about by decreased elimination in the faeces. Compared with the faecal excretion, the effects of variations in the urinary excretion on the calcium and phosphorus balances are unimportant.

Unfortunately it is impossible to differentiate the faecal excretion into the unabsorbed portion and the excreted portion; and consequently there is no direct evidence to settle the question as to whether the oils merely cause an increased absorption of these minerals, or cause an increased retention after they are absorbed.

It has been generally believed that calcium salts are excreted chiefly by the large intestine. The fact that calcium salts, when injected intravenously, appear in the intestine, seems to support this view (a review of this work is given by Von Noorden [1907]).

The results of the recent interesting studies of Telfer [1922], however, make it appear doubtful whether, normally, calcium or phosphorus is excreted into the intestine. He regards the mineral matter of the faeces in his experiments as chiefly, if not entirely, unabsorbed residues.

If the mineral matter in the large intestine is to be regarded as unabsorbed residues, then the effect of oil is exercised in the alimentary tract. The increased absorption must however be due to something more than a mere physical or chemical change in the contents of the intestine, because in Exp. 1, where the post-period was continued for 12 days, increased retention of calcium was continued after the elimination of the oil from the diet. Indeed, retention of calcium was greater in the days 7 to 12 of the post-period than during the administration of the oil.

Comparative effect of the three oils.

A comparison of the effects of the three different oils is of interest. In Exp. 1 when the pre-period was short, and the three animals were still

retaining a considerable amount of calcium and phosphorus, the oils appear about equally potent in increasing the retention of both minerals.

In Exps. 2 and 3, when the animals had got into a pathological condition, as shown by the negative calcium balances, cod-liver oil was the most potent in increasing the assimilation and retention of the minerals. Of the other two, olive oil appears from a comparison of pigs Nos. 68 and 670 with pig. No. 671, to be greater in its action than linseed oil. On the other hand, there was a marked improvement in the appetite of the animals when given linseed oil or olive oil, but the introduction of cod-liver oil resulted in a depression of the appetite. Hart, Steenbock and Hoppert [1922] noted a similar effect of cod-liver oil on the appetite of goats.

The fact that olive oil and linseed oil, which contain little or no fat-soluble A, are almost as effective in stimulating the assimilation and retention of calcium and phosphorus as the vitamin-rich cod-liver oil, indicates that the action on the metabolism of the minerals is not due, entirely at all events, to the vitamin A.

This conclusion is supported by the work of McCollum, Simmonds, Becker and Shipley [1922] who tested a number of fats for their power to stimulate healing in rickets. They found that cod-liver oil oxidised for 12 hours to destroy fat-soluble A, still retained its power to cause the deposition of calcium in the bones of young rats suffering from rickets. A similar result was got with coconut oil, which is also deficient in vitamin A.

It is most probable that oil *per se* has an influence on the absorption of minerals from the intestine, though the above workers found that olive oil fed to rats suffering from rickets did not raise the efficiency of the tissues in utilising calcium. In the light of our results it is probable that a positive result would have been obtained by these workers if the oil had been introduced earlier, or if the experiment had continued over a longer period. Unfortunately in these experiments with rats there are no data to show the actual mineral balances.

In our experiments, when the animals had been showing a negative calcium balance for some time, the response when receiving linseed oil was not so rapid as in the case of animals still assimilating and retaining these minerals (cf. pig No. 671, Exp. 3 with pig No. 24 of Exp. 1).

The results obtained here make it clear that the effect produced on a growing animal by the introduction of cod-liver oil cannot be ascribed to the vitamin present in the oil.

NOTE. It will be seen from the tables and graphs that the nitrogen metabolism of each pig was also followed. These results seem to indicate some difference in the action of the three oils on the nitrogen metabolism. This point is being further investigated, and it is hoped to discuss the combined results in a future communication.

CONCLUSIONS.

1. Pigs confined in metabolism cages and fed on a grain ration show a progressively decreasing capability of absorbing or retaining calcium and phosphorus.

2. The introduction of cod-liver oil, linseed oil or olive oil into the grain ration leads to increased assimilation and retention of both minerals.

3. When the animal is still retaining appreciable amounts of calcium and phosphorus, the three oils are about equally potent in their action.

4. When the animal shows a negative calcium balance, both cod-liver oil and olive oil rapidly convert the negative calcium balances to positive balances. Linseed oil is much slower in its action.

5. When the animal shows a negative calcium balance the introduction of cod-liver oil into the ration causes digestive disturbances.

6. The beneficial effect of an addition of oil to a grain ration on the calcium and phosphorus metabolism appears to be largely due to the oil *per se* irrespective of its content of vitamin A.

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LXXXIX. CARBOHYDRATE AND FAT METABOLISM IN YEAST.

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(Received August 21st, 1923.)

PART I. THE FORMATION OF FAT FROM NON-NITROGENOUS SUBSTANCES OTHER THAN SUGARS.

FORTY-FIVE years ago Nägeli [1878, 1879] examined the production of fat by moulds and yeasts and found that the fat content of these organisms could be increased from 5 % to 12 % when they were grown in a well-aerated medium rich in carbohydrate and poor in nitrogen. During the war Lindner [1922] and his co-workers applied similar conditions of growth to the organism *Endomyces vernalis*, and the fat content of this was so successfully raised that it was used in Germany to provide rations both of fat and protein. Lindner also made a histological investigation of yeast cells grown under similar conditions of aeration in a medium rich in carbohydrate and poor in nitrogen. He describes as the first stage of fat formation a granular appearance of the plasma, the granules coalescing gradually to form fat globules, which could be stained with appropriate reagents, *e.g.* osmic acid, Sudan III or alkanna. Solutions of sugar and of alcohol were found to be suitable media from which the yeast cell could increase its quantity of stored fat. Superficial cultures of yeast cells were also found to become laden with fat globules when submitted to the action of alcohol vapour. Berzfeld [1922] described the power of the yeast cells to stain with alkanna and Sudan III as a criterion of age, quality and degeneration and found it was not till the third or fourth generation that the fat in the yeast cell could be shown by staining. There are also a number of patents dealing with the changes produced by different media on the fat content of yeast. Quantitative data have been however almost entirely lacking and even the relative efficiency of sugar and alcohol as sources from which the yeast cell could produce fat does not seem to have been determined.

Nägeli was aware that the direct extraction of dried yeast by ether did not remove all the fat it contained, but subsequent workers do not appear to have recognised this. In an earlier communication by one of us it was shown that the amount of fat which could be obtained from a sample of yeast depended on the method of extraction used [Smedley MacLean, 1922]. The proportion of the total fat present which could be extracted directly from dried yeast in a Soxhlet apparatus by ether also varied according to the previous history of the yeast. From yeast previously incubated in a well-oxygenated solution of sugar only a small proportion of the fat it contained was separated by direct extraction, but if the yeast was first hydrolysed by boiling it for two

hours with normal acid and the residue dried and extracted with ether a much larger quantity of fat was obtained than by the direct extraction process; the acid value of the fat was only slightly raised, showing that hydrolysis had only taken place to a very small extent. If the fat was estimated in an old sample of yeast both by ether extraction of the dried yeast and by extraction of the dried residue after hydrolysis there was much less difference between the results obtained by the two methods than in the case of the recently incubated yeast described above. The fat content of all samples of yeast was however found to be higher when determined after hydrolysis of the yeast.

The hydrolysis method has also the advantage of allowing the carbohydrate content of the yeast to be determined at the same time in the filtrate so that the relation between the fat and carbohydrate contents of the yeast may be conveniently studied. By the method of acid hydrolysis any lipin present in the fat would be partly decomposed and glycerophosphoric acid separated, so that if any lipin were present the fat obtained by direct extraction would lose a fraction of its weight during the hydrolysis.

It is clear that care must always be taken to differentiate between a real and an apparent increase of fat in the cell. Since the fat when first formed in the yeast cell appears to be in a condition in which it cannot be directly extracted by ether, the effect of incubating the yeast in a certain medium might conceivably be to produce an apparent increase of fat by altering the state in which the fat is held in the cell and rendering it capable of being extracted by solvents. If the yeast be first heated with dilute acid as described this difficulty is eliminated.

It seemed to us that it would be of interest to obtain quantitative data as to the amount of fat stored in yeast when the yeast is incubated in solutions of various non-nitrogenous organic substances and we investigated therefore the action in this respect of the following:

- (1) simple organic substances such as alcohols and the sodium salts of fatty acids;
- (2) the sugars: lactose, glucose, fructose, sucrose and maltose;
- (3) sugar solutions with the addition of alkali phosphates.

METHOD OF EXPERIMENT.

Samples of the yeast were weighed out for the determination of moisture and nitrogen and four quantities each of 12.5 g. were also weighed; two of these samples were hydrolysed by boiling each with 100 cc. of *N* HCl for two hours under a reflux condenser; the mixture was filtered and the residue well washed with hot water; to the filtrate and washings 80 cc. of *N* NaOH were added and about 30 cc. of "dialysed iron" and the mixture was made up to 1 litre. After filtering, the reducing power of the filtrate was determined by Bertrand's method and the total reducing carbohydrate present after hydro-

lysis calculated as glucose for the purpose of comparison. The residue of yeast cells after boiling with the acid was air dried over-night on the filter and then extracted with ether for 48 hours in a Soxhlet apparatus. After evaporation of the ether the residue was taken up with dry ether, filtered into a weighed flask, the ether evaporated and finally the ether-soluble substance dried to constant weight in a vacuum desiccator. The two remaining quantities of 12.5 g. of yeast were added each to a litre of the solution to be tested contained in a Winchester quart bottle. Into one of these a tube was fitted through the cotton-wool plug and after placing in the incubator at 25–26°, a current of oxygen was passed through for 45 hours; the other was placed in the incubator, no oxygen being passed through. At the end of the experiment the contents of each were centrifuged and the yeast filtered on a Buchner funnel, well washed and weighed. Samples were taken for the determination of moisture and nitrogen and the remainder hydrolysed and treated exactly as described for the original yeast.

The substance soluble in the dry ether was regarded as fat; the acid value of the fat obtained in this way was found to be slightly higher than that of the fat directly extracted by ether from the dried yeast. It is possible that the slight increase is due to acid liberated from the lipins present. The yeast sterol, previously identified as ergosterol [Smedley MacLean and Thomas, 1920], is always present with the fat. In our earlier experiments the pressed yeast used was supplied by a yeast dealer, but the results obtained were not consistent; the age of the yeast seemed to be an important factor since the power of a specimen of pressed yeast to form fat fell off rapidly when the yeast was kept in the cold room for one or two days before being used. We subsequently worked with an ale yeast from 84 to 110 hours old supplied directly from a brewery where we could rely on the yeast being grown under approximately constant conditions; this yeast we found contained appreciably less sterol than that used in our earlier experiments.

THE EFFECT ON THE FAT CONTENT OF YEAST PRODUCED BY INCUBATING
IT IN SOLUTIONS OF SIMPLE ORGANIC SUBSTANCES.

The effect of incubating yeast for 45 hours in water at 25° with and without oxygenation was first ascertained as a basis of comparison for future experiments. We found that 12.5 g. of brewery yeast incubated for 45 hours at 25° in a litre of water and oxygenated throughout this period lost about two-thirds of the carbohydrate originally present and about a seventh of the protein; the quantity of fat however increased by from 50 to 100 % of that originally present. If the oxygen was not passed there was slightly less loss of carbohydrate and of protein and no increase of fat. These experiments confirm the results previously arrived at [Smedley MacLean, 1922] that a yeast with a high carbohydrate content loses carbohydrate and gains fat when incubated in oxygenated water. The specimens of pressed yeast ex-

amined at an earlier date had been kept for some time and did not show the increase of fat after incubation in water, but as stated above we found that the fat-forming power of a yeast rapidly fell off on keeping the yeast in the cold room.

In subsequent experiments carried out with 12.5 g. of yeast containing approximately 0.1 g. of fat we have regarded increases of fat up to 0.1 g. as capable of being formed from materials originally present in the cell since this increase could be obtained by incubating the yeast in oxygenated water. If the amount of fat was increased beyond this we have attributed it to the influence of the substance dissolved in the medium.

The substances investigated were the alcohols, methyl, ethyl, propyl, *iso*-propyl, butyl and *iso*-amyl, glycol and glycerol; acetone, acetaldehyde and the sodium salts of the following acids, formic, acetic, butyric, lactic and pyruvic. The method of procedure was to make up a solution of the substance to be tested, generally of decinormal strength and to incubate the yeast in this as described above.

We found we could divide these substances into three classes:

(a) *Those producing a definitely inhibitory effect*, no increase of fat being observed or an amount so small as to be within the region of experimental error. Under this heading we include methyl, propyl, butyl and *iso*-amyl alcohols, that is to say that all the normal fatty alcohols tested, with the exception of ethyl alcohol, appear to be definitely injurious to the formation of fat when supplied in decinormal solution, less fat being found than when the yeast was incubated under similar conditions in water. We found also that acetaldehyde supplied in 0.6 % concentration produced little change in the fat and carbohydrate content; it appeared to be injurious to the yeast and there was a marked increase in the loss of protein. These results contrast with those obtained by Haehn [1923] in his recently published paper on *Endomyces vernalis*, where a marked increase of fat was observed when the organism was supplied with acetaldehyde in a concentration of 1 %, alkali phosphates being also present in the solution.

(b) *Substances, solutions of which behave similarly to water* and which may therefore be assumed to have no influence on the production of fat. Decinormal solutions of the sodium salts of formic, propionic and butyric acids, of *iso*-propyl alcohol and acetone, glycol in 0.5 % and glycerol in both 0.5 and 5 % solutions belong to this class. Small increases of fat were recorded quite comparable with those observed when the yeast was incubated in water and the amount of carbohydrate found at the end of the period of incubation was also of the same order in both cases.

(c) *Substances which produce an increase of fat greater than that observed after incubation of yeast in water*. To this class belong ethyl alcohol, and the sodium salts of acetic, pyruvic and lactic acids.

Ethyl alcohol. Our experiments showed that if yeast were incubated for 45 hours in a 0.5 or 0.6 % solution of ethyl alcohol, the increase of fat in the

yeast at the end of the incubation period was more than after incubation in water for the same time; in one experiment only did we get an increase of fat comparable with that obtained after yeast had been incubated in a sugar solution, but in six other experiments carried out under as far as we knew similar conditions the increase was very much smaller. If we increased the percentage of alcohol in the medium, the amount of fat stored showed no corresponding increase but a diminution.

Lindner and Unger [1919] have described the effect of alcohol vapour on different brewery yeasts and have shown that it brings about the deposition of globules of oil in the cell. It is possible that this result may be partly due to a change in the state of fat in the cell whereby the separation of the fat already present in the cell is affected. Also, that the microscopic changes which Lindner himself has described, the preliminary granulation of the cell plasma gradually followed by the collection of the granules in globules, may be hastened by the effect of the alcohol vapour. Lindner [1922] found that both with brewery yeasts and with *Endomyces vernalis* the assimilation of alcohol leads to the production of fat. In our experiments with brewery yeast alcohol is very much less effective as an aid to the formation of fat than any of the fermentable sugars. In 0.5 % solution, ethyl alcohol is as effective as a 0.5 % sugar solution in producing fat, but the amount of fat formed does not increase when the concentration of alcohol in the solution is increased and alcohol cannot therefore be put into the same category as the sugars when classified according to its fat-forming power.

Sodium acetate we found rather more effective as a fat former than ethyl alcohol, but again when the concentration of the acetate was increased above decinormal there was a diminished increase of fat. The same held also for sodium lactate and for sodium pyruvate.

These four substances can certainly be classed together as having a distinct though small influence on the production of fat. We do not however feel justified in saying that from these experiments there is any satisfactory evidence that any one of these substances can be regarded as an intermediate stage in the process of fat formation.

The increase of fat observed after incubating yeast in oxygenated water clearly proves that fat is formed from the carbohydrate held in the cell, since the loss of protein is insufficient to account for it. It may be that by the assimilation of such substances as ethyl alcohol or sodium acetate the carbohydrate storage is directly influenced and the fat formation is only indirectly influenced, and it is interesting to note that in these cases the carbohydrate content of the yeast is higher after incubation in the oxygenated than in the non-aerated solution and the amount stored may even exceed that present in the original sample of yeast. Except for one experiment with alcohol in which an abnormally high fat content was obtained and which we have never been able to repeat, the largest increase of fat was obtained after incubation in oxygenated sodium acetate solution where we got an increase of from 0.1

Table I.

Medium	Original yeast				Oxygenated yeast			Non-oxygenated yeast		
	Age of yeast, Hours	Carbo-hydrate g.	Fat g.	Protein g.	Carbo-hydrate g.	Fat g.	Protein g.	Carbo-hydrate g.	Fat g.	Protein g.
Water	88	1.21	0.0897	1.31	0.39	0.1420	1.12	0.50	0.0912	1.11
	110	0.75	0.1042	1.20	0.30	0.1590	1.02	0.31	0.0932	1.08
	70	1.52	0.1039	1.15	0.57	0.1978	0.98	0.65	0.1051	1.05

Substances, incubation in solutions of which produces less increase of fat than incubation in water at 25°.

Alcohols:

Methyl M/10	106	0.64	0.1229	1.29	0.27	0.1463	1.26	0.33	0.1200	—
Propyl M/10	83	0.72	0.1027	1.25	0.30	0.1159	1.08	0.37	0.1049	1.14
Butyl M/10	102	0.70	0.0817	1.40	0.32	0.0898	1.27	0.31	0.0784	1.18
iso-Amyl M/10	108	0.65	0.1107	1.37	0.28	0.1096	1.18	0.31	0.1127	1.18

Acetaldehyde:

0.1 %	108	0.91	0.1050	1.46	0.37	0.1500	1.33	0.53	0.1110	1.41
0.6	78	0.99	0.0978	1.26	0.92	0.0890	0.82	0.87	0.0906	0.75

Substances, solutions of which act like water.

Na formate M/10	86	1.52	0.0918	1.29	0.56	0.1841	1.17	0.46	0.0935	1.30
Na propionate M/10	62	1.20	0.1176	1.22	0.32	0.1856	1.10	0.36	0.1150	1.09
Na butyrate M/10	—	0.47	0.0981	1.29	0.27	0.1849	1.15	0.37	0.1187	1.17
	84	1.39	0.0920	1.30	0.70	0.1625	1.20	0.74	0.1033	1.24
iso-Propylalcohol M/10	106	0.98	0.0893	1.32	0.38	0.1745	1.01	0.37	0.0968	1.20
Acetone M/10	85	1.22	0.1006	1.43	0.57	0.1607	1.26	0.65	0.1040	1.33
Glycol 0.5 %	85	1.29	0.0920	1.35	0.39	0.1497	1.21	0.41	0.0816	1.20
" 0.5	101	0.74	0.1092	1.44	0.36	0.1404	1.29	0.42	0.1010	1.34
Glycerol 0.5 %	84	1.15	0.1228	1.20	0.46	0.2086	1.10	0.47	0.1073	1.18
" 5.0	72	1.19	0.1167	1.23	0.43	0.1997	1.11	0.33	0.1148	1.13

Substances, solutions of which produce marked increase of fat.

Ethyl alcohol:

0.5 %	103	1.27	0.0862	1.08	0.53	0.2610	1.24	0.46	0.1032	1.22
0.5	83	0.83	0.1065	1.36	0.50	0.2417	1.26	0.44	0.1144	1.22
0.5	86	0.89	0.1065	1.24	0.71	0.5221	1.16	0.54	0.0984	1.20
0.5	83	1.12	0.1163	1.23	0.66	0.1889	1.19	0.74	0.1254	1.20
0.64	130	1.03	0.1110	1.34	0.43	0.2636	1.22	0.38	0.1125	1.22
2.0	106	0.97	0.1122	1.34	0.41	0.2225	1.24	0.33	0.1064	1.21
4.0	76	1.30	0.1140	1.24	0.47	0.1952	1.09	0.47	0.1032	1.15

Sodium acetate:

0.6 %	—	0.51	0.1816	1.48	1.01	0.3930	1.35	0.40	0.1882	1.45
	96	0.60	0.1084	1.33	0.34	0.3475	1.16	0.22	0.1062	1.19
	84	1.21	0.0846	1.32	1.10	0.2136	1.22	0.50	0.0897	1.21
1.0	83	1.12	0.1034	1.23	0.41	0.2182	1.11	0.43	0.1059	1.35
2.0	106	1.14	0.0927	1.36	0.34	0.2172	1.25	0.42	0.0945	1.26
5.0	105	0.80	0.1126	1.27	0.28	0.1417	0.93	0.26	0.1049	0.86

Sodium lactate:

0.90 %	82	1.10	0.1208	1.31	0.42	0.2470	1.23	0.30	0.1180	1.09
	107	0.80	0.1058	1.31	0.39	0.1664	1.15	0.37	0.1080	1.08
2.0	84	1.31	0.1035	1.23	0.51	0.2113	1.16	0.43	0.1070	1.20

Sodium pyruvate:

0.88 %	82	1.31	0.0945	1.29	0.53	0.2291	1.19	—	—	—
	94	0.89	0.1026	1.39	0.47	0.2204	1.20	—	—	—
	96	1.14	0.1006	1.33	0.60	0.2461	1.32	0.69	0.1185	1.33
	81	1.05	0.0932	1.31	0.40	0.2270	1.24	0.38	0.1153	1.17

to 0.35 g., an increase of about 10 % on the dry weight of the yeast. Since in some of the acetate experiments there was at the end of the experiment an actual increase in the amount of the carbohydrate stored in the cell, it

follows that the cell was able to build up carbohydrate from the acetate supplied and some of the newly formed carbohydrate may have been converted to fat.

It may be that we cannot present any simple intermediate substance to the cell under suitable conditions for the cell to make use of it; it does not necessarily follow that these substances may not occur in the cell as intermediate substances. It is interesting to note that Stephenson [1922] found that sodium acetate exerted a beneficial effect on fat formation in the Timothy Grass *Bacillus*.

PART II. THE RELATIVE EFFICIENCY OF THE DIFFERENT SUGARS IN PRODUCING STORAGE OF CARBOHYDRATE AND OF FAT.

When yeast is incubated in an oxygenated solution of fermentable sugar, the fat content is increased.

In stating our results we have recorded the actual amounts of fat, carbohydrate and protein in the yeast before incubation and compared these with the amounts present when the same weight of the yeast has been incubated in a solution of the substance under investigation. A comparison of the percentages of fat and carbohydrate found in the samples of yeast before and after incubation calculated on the dry weight of the yeast may give rise to misleading conclusions. No nitrogen being present in the nutrient solutions, an increase in weight must be due to an increase in the amount of fat or carbohydrate present. If the latter be increased, although no fat may have been lost, the percentage of fat will be diminished and similarly a diminution of the carbohydrate content produces an apparent increase of fat when expressed as fat percentage although the total amount of fat present may be less than at the beginning of the experiment.

The method of carrying out the experiments was the same as that described above for the simpler carbon compounds (cp. p. 721). The pressed yeast with which we worked showed comparatively small variations in the percentage of fat it contained. The 12.5 g. used for each experiment contained approximately 0.1 g. fat, the amount in the vast majority of samples lying between 0.08 and 0.1 g. and the results of the experiments were practically unaffected by these small differences.

The variations in the carbohydrate contents of the original pressed yeast were very much wider and seem to influence to some extent the final figures for the carbohydrate when the yeast is incubated in a 0.5 or 1.0 % solution of sugar. In stronger solutions the influence does not seem to be perceptible. A survey of the accompanying tables will however show that the amounts of carbohydrate contained in a fixed weight of yeast after it has been incubated in a sugar solution are remarkably constant for any given concentration of solution and must depend mainly on this factor.

With regard to the nature of the fat formed it appears to be closely similar to that present in the original yeast; it shows the same power of remaining

concealed in the cell until after the yeast has been hydrolysed with dilute acid, which has already been described. The saponification number of the fat from different samples lay between 139 and 186 and the Hübl Iodine Values varied considerably, the limits being from approximately 80 to 132. These differences may be partly explained by variations in the amount of ergosterol present and partly perhaps by oxidation, as the newly formed fat

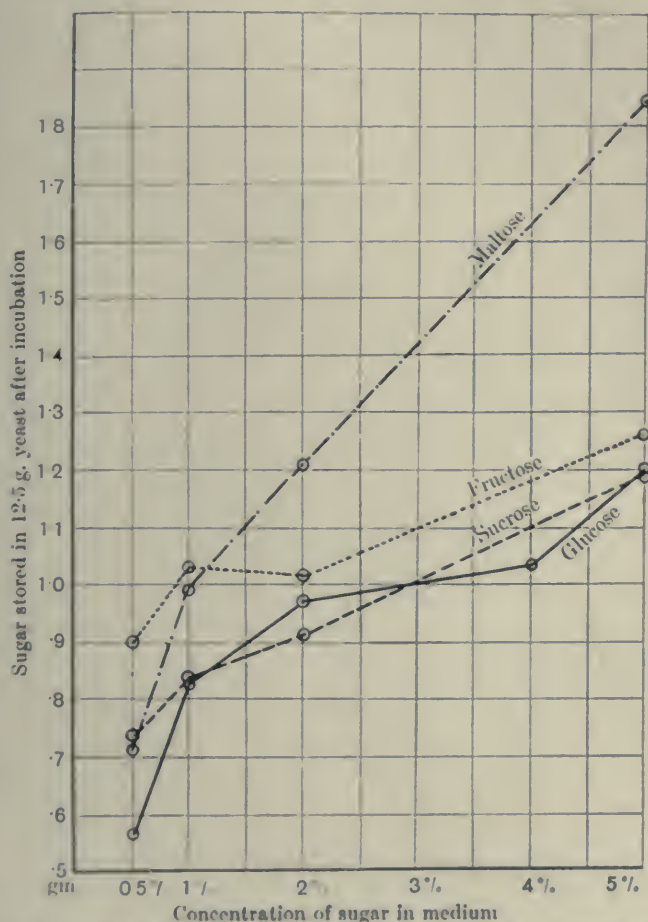


Fig. 1. Curves showing relation of concentration of sugar in oxygenated medium to amount of carbohydrate stored by yeast.

seemed very susceptible to oxidation. We were unable to detect any consistent differences between the fat formed under different conditions and have therefore not recorded these figures in detail.

The sugars investigated were lactose, glucose, fructose, sucrose and maltose.

Lactose. Of these the only sugar which was not fermented by the yeast was lactose and it alone appeared to exercise no influence on the storage of either carbohydrate or fat within the yeast cell. A 5% solution of lactose

behaved very much like water; yeast when incubated in it for 45 hours at 25° showed a small storage of fat and a considerable loss of carbohydrate if the solution had been oxygenated and no storage of fat and rather less loss of carbohydrate in the yeast from the non-oxygenated medium.

Glucose, fructose and sucrose. These three sugars closely resembled each other in their influence on the storage of both carbohydrate and fat. After

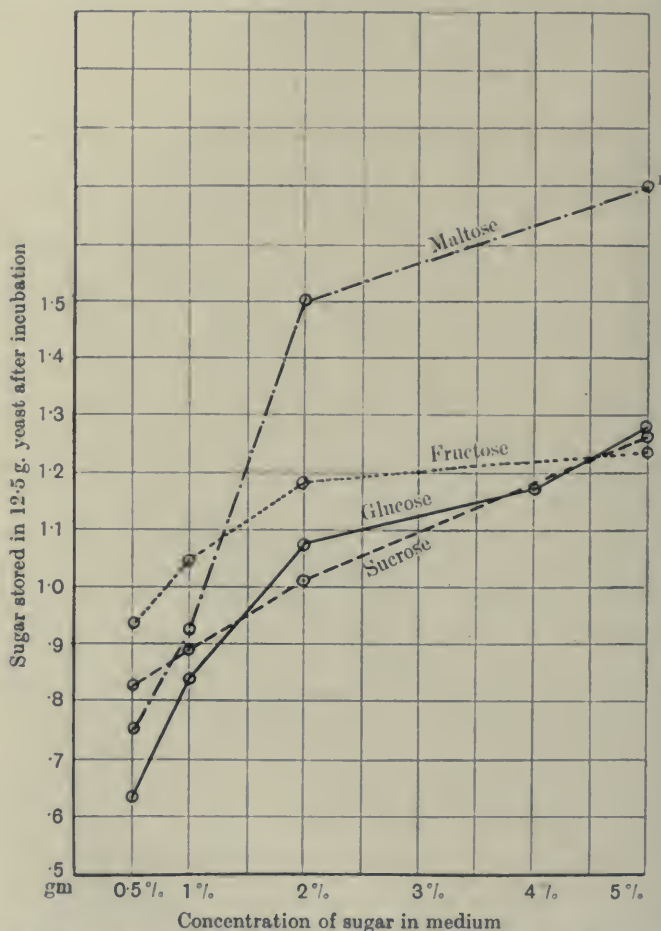


Fig. 2. Curves showing relation of concentration of sugar in non-oxygenated medium to amount of carbohydrate stored by yeast.

incubating a fixed quantity of yeast in a sugar solution the amount of carbohydrate stored depended chiefly on the concentration of sugar in the solution in which the yeast had been incubated. The divergences between the three sugars were most apparent in the very dilute solutions; it was here that the amount of carbohydrate present in the original sample of yeast appeared to exercise some influence, for if equal weights of a yeast with a high and a yeast

¹ The mean of three experiments gave 1.6 g. maltose; as one of these values was abnormally low the number 1.7 has been used in the diagram (cp. Table II).

with a low carbohydrate content were incubated in a 0.5% or in a 1% solution of the same sugar, the final carbohydrate content was generally higher in

Curves showing relation between increase of fat after incubation in sugar solutions and concentration of solution.

Oxygenated

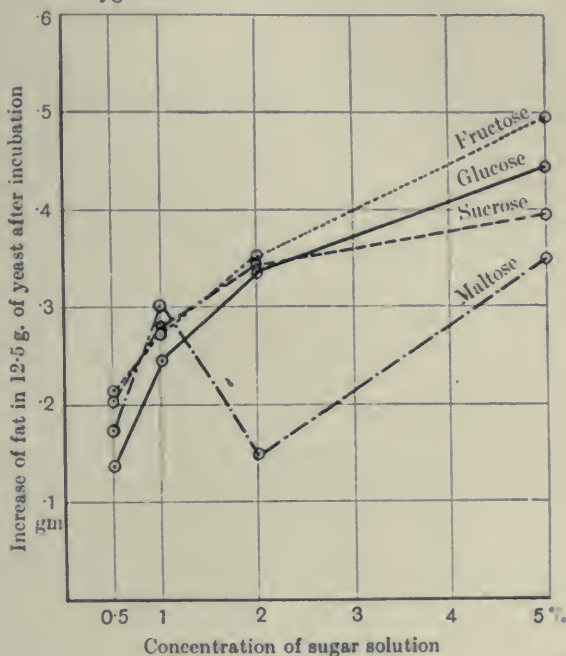


Fig. 3.

Not oxygenated

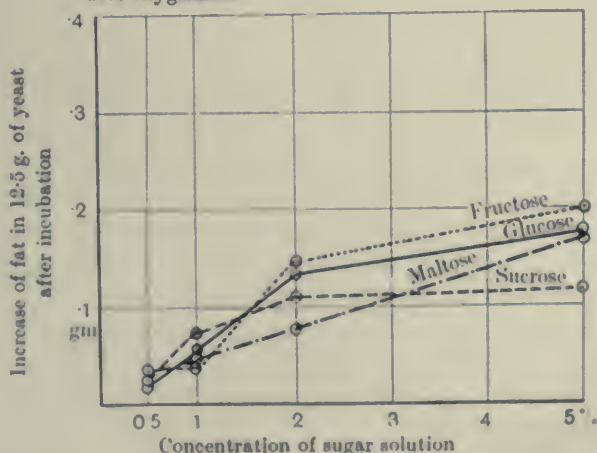


Fig. 4.

the yeast which had the greater content originally. But even with very considerable differences in the carbohydrate contents of the original samples

of yeast, the final amounts of carbohydrate present fell within comparatively narrow limits for any given concentration of sugar.

As the concentration of the solution was increased from 0.5 to 5.0 % the amount of stored carbohydrate rose more sharply at first from 0.5 to 2 %, more gradually from 2 to 5 %. This is apparent from a survey of the curves shown in Figs. 1 and 2.

Fructose in concentrations of from 0.5 to 2 % seems to produce a rather greater storage of carbohydrate than either glucose or sucrose. On the whole we are inclined to think that the figures obtained may possibly represent a real difference in the action of these sugars, though the difference is a small one; the carbohydrate content of yeast after incubation in fructose solutions is higher than that of yeast incubated in glucose and sucrose solutions both with and without oxygenation; and in both cases the difference disappears when the concentration of the sugar rises to 5 %. The effect of oxygenating the sugar solution in which the yeast is incubated is slightly to reduce the carbohydrate content. This result is not invariable but occurs in the majority of cases.

The increase of fat stored up after incubation in an oxygenated solution of glucose, fructose or sucrose is practically the same (Figs. 3 and 4). Fructose gives slightly more fat, but the differences are so small that it is difficult to say whether any significance should be attached to them. The fat-forming power of yeast seems to vary considerably in the different samples and the numbers denoting the amounts of fat stored for any given concentration of sugar in the medium are not as constant as the corresponding numbers denoting the amounts of carbohydrate. For instance four or five experiments were carried out with 5 % solutions of the three sugars; the mean value for the fat content of the yeast after incubation in the fructose solution (oxygenated) was quite appreciably higher than that from the 5 % oxygenated solutions of either glucose or sucrose, but the highest value in any of the experiments was in one of the glucose solutions. We think therefore that though the numbers obtained are suggestive we cannot say with certainty that there is any pronounced difference in the fat-forming powers of these three sugars. With all three the amount of fat deposited in the yeast which has been incubated in a solution of the sugar varies with the concentration of sugar in the medium; there is a gradual flattening in the curve showing the relation of the increase of fat stored to the concentration of sugar, the rate of increase of the fat falling off as the concentration of sugar rises.

The effect of oxygenating the solution is very marked; after oxygenation the fat content is about double as much as in a yeast grown in a similar solution without oxygenation.

The most marked difference between the conditions affecting the storage of fat and of carbohydrate is that, while oxygenation of the solution in which the yeast is incubated diminishes very slightly the amount of carbohydrate in the yeast, it largely increases the formation of fat.

Maltose. Comparison of maltose with the sugars whose action has already been described shows that solutions of maltose of 1 % and over produce markedly greater storage of carbohydrate than do solutions of fructose, glucose or sucrose of similar strength.

Rose [1910] working with *Endomyces vernalis* showed that maltose was a better source of carbon than glucose in a medium which contained also magnesium sulphate, potassium phosphate and asparagine. Lindner and Saito [1910] tested the assimilating power of a very large number of yeasts for various sugars and came to the conclusion that maltose was the best assimilated; Kita's [1914] experiments also confirm this view. Kluver [1913], starting from the standpoint that these results are not in accordance with the view that all substances before absorption are broken up into the simplest "bricks," repeated Lindner's experiments weighing his yeast after drying at 105° and found definite increases when Kahlbaum's maltose was used, smaller or no increases when the maltose had been first purified by recrystallisation from 80 % alcohol. He found also that the original Kahlbaum sample of maltose contained 0.22 % nitrogen which was reduced after purification to only 0.01 %; he therefore argued that the asparagine of the original solution provided an insufficient nitrogen supply and the effect of the maltose was due to the nitrogenous impurities it contained. Our experiments differed from Lindner's in that our media contained no nitrogen and we measured directly the carbohydrate stored; determinations of the nitrogen in the yeast samples were made both before and after the incubation, and within the limits of the experimental error the nitrogen content was unchanged. The nitrogen contents of the sucrose and maltose used in these experiments were identical. The experiments now carried out seem to us to show quite conclusively that *when yeast is incubated in maltose solutions of from 1 to 5 % there is much greater carbohydrate storage in the yeast cells than when solutions of glucose, fructose or sucrose are used.*

Maltose solutions however show themselves on the whole to be rather less effective in producing an increase of fat than are solutions of either glucose, fructose or sucrose.

Another point observed while carrying out these experiments is perhaps worthy of notice. After yeast had been incubated in 2 % and in 5 % sucrose solution the weight of the pressed yeast was very much smaller than in the case of yeast from the corresponding solutions of any other sugar. The yeast seemed to have undergone a certain amount of plasmolysis, the dry weight of the yeast, and the amounts of protein, carbohydrate and fat being the same, but the weight of the pressed yeast being only about two-thirds that of the yeast after incubation in solutions of the other sugars.

As the different samples of yeast showed much variation in their power of forming fat, equal quantities of the same sample of yeast were incubated at the same time under similar conditions in a 2 % solution of glucose and in a 2 % solution of one of the sugars, fructose, sucrose or maltose, so that a direct

Table II.

Sugar %	Temp. °C.	Age of yeast. Hours	Original yeast			Incubated in oxygenated solution			Incubated in non-oxygenated solution		
			Fat g.	Carbo-hydrate g.	Protein g.	Fat g.	Carbo-hydrate g.	Protein g.	Fat g.	Carbo-hydrate g.	Protein g.
Glucose:											
0.5	25.5	84	0.1057	1.00	1.36	0.1810	0.55	1.27	0.1262	0.62	1.19
"	Lab.	110	0.1017	1.00	1.39	0.1667	0.57	1.26	0.1040	0.65	1.40
"	24.5	109	0.1012	0.65	1.46	0.2720	0.41	1.25	0.1231	0.42	1.37
"	25	106	0.1040	1.02	1.23	0.1556	0.50	1.12	0.1318	0.66	1.16
"	25	85	0.0646	1.12	1.24	0.3660	0.79	1.15	0.0842	0.78	1.15
1.0	25	110	0.1051	0.82	1.31	0.3358	0.92	1.35	0.1413	0.75	1.28
"	25.5	89	0.1041	1.18	1.26	0.4618	0.83	1.19	0.1830	0.90	1.21
"	24	85	0.0880	1.06	1.14	0.2432	0.71	1.15	0.1457	0.86	1.13
2.0	25.5	89	0.0976	1.07	1.34	0.4220	0.96	1.17	0.2326	0.80	1.27
"	25.5	84	0.1041	1.47	1.18	0.4805	0.84	1.15	0.2350	0.99	1.09
"	25	—	0.0905	1.37	1.15	0.3610	1.03	1.06	0.2220	1.27	1.08
"	25.5	105	0.0805	1.18	1.18	0.3703	0.98	1.17	0.2074	1.22	1.16
"	25	81	0.0785	1.53	1.16	0.4770	1.04	1.11	0.2096	1.04	1.07
"	25	103	0.0890	0.83	1.26	0.4818	0.84	1.24	0.2123	1.17	1.18
"	25	84	—	—	—	0.5095	0.98	1.20	0.2498	0.98	1.20
5.0	27.5	106	0.0985	0.70	1.33	0.5286	1.18	1.31	0.2645	1.20	1.27
"	25.5	84	0.1055	0.69	1.44	0.3956	1.37	1.35	0.2190	1.40	1.29
"	25.5	84	0.9870	1.65	1.13	0.7110	1.08	1.00	0.3495	1.24	0.99
Fructose:											
0.5	25.5	86	0.1018	1.52	1.22	0.1853	0.99	1.19	0.1306	1.01	1.22
"	25	76	0.1042	1.18	1.22	0.4015	0.88	1.21	0.1355	0.99	1.14
"	24.5	84	0.0800	1.46	1.19	0.3477	1.09	1.15	0.1183	1.08	1.15
"	25	88	0.0677	1.03	1.37	0.2380	0.77	1.26	0.1077	0.80	1.26
"	25	85	0.0646	1.12	1.24	0.3404	0.77	1.19	0.0993	0.78	1.21
1.0	25	82	0.1056	1.13	1.22	0.3222	0.85	1.16	0.1248	1.00	1.17
"	25.5	—	0.0925	1.17	1.26	0.4245	1.21	1.23	0.1414	1.08	1.14
2.0	24.5	82	0.1037	1.09	1.31	0.3658	0.97	1.20	0.2327	1.22	1.13
"	24.5	82	0.1037	1.09	1.31	0.4258	1.00	1.25	0.2066	1.22	1.10
"	25	83	0.0885	1.26	1.24	0.5465	1.08	1.14	0.2574	1.18	1.10
"	25	82	0.1056	1.13	1.22	0.4956	0.91	1.17	0.2305	1.08	1.15
"	25.5	—	0.0890	0.83	1.28	0.4729	0.93	1.23	0.3007	1.12	1.23
"	25	80	0.0848	1.45	1.18	0.3794	1.12	1.13	0.2395	1.34	1.17
5.0	25.5	86	0.0979	1.00	1.28	0.5575	1.13	1.49	0.2574	1.24	1.45
"	"	118	0.0977	0.95	1.10	0.6266	1.35	1.07	0.3358	1.35	1.01
"	"	96	0.1046	0.82	1.43	0.5360	1.29	1.37	0.2254	1.10	1.36
Sucrose:											
0.5	25	84	0.0941	1.31	1.23	0.3457	0.68	1.14	0.1252	0.80	1.22
"	"	87	0.0910	1.24	1.25	0.2874	0.77	1.18	0.1226	0.87	1.15
"	"	82	0.0835	0.84	1.33	0.2516	0.73	1.15	0.0891	0.80	1.17
1.0	"	106	0.1022	1.22	1.29	0.2810	0.96	1.21	0.1462	0.99	1.20
"	"	86	0.0970	1.47	1.20	0.4405	0.71	1.18	0.1927	0.86	1.15
"	"	91	0.0706	0.77	1.37	0.3860	0.83	1.22	0.1457	0.82	1.23
2.0	"	106	0.1050	1.13	1.30	0.4355	1.03	1.27	0.2107	1.12	1.23
"	"	81	0.0785	1.53	1.16	0.4704	0.80	1.09	0.1996	0.93	1.09
"	"	84	0.0943	1.12	1.23	0.3980	0.90	1.16	0.2012	0.99	1.16
5.0	"	84	0.0815	1.23	1.25	0.3803	1.18	1.13	0.2037	1.40	1.15
"	"	85	0.0998	1.25	1.30	0.3500	1.30	1.16	0.1750	1.40	1.15
"	"	106	0.0928	1.00	1.24	0.6008	1.22	1.10	0.2522	1.18	1.10
"	"	96	0.0888	1.10	1.28	0.6097	1.07	1.18	0.2398	1.06	1.12
Maltose:											
0.5	25.5	87	0.1040	1.00	1.30	0.3041	0.71	1.24	0.1350	0.77	1.25
"	24.5	85	0.0867	1.02	1.32	0.2433	0.72	1.30	0.0935	0.72	1.21
1.0	25.5	110	0.1001	1.00	1.25	0.2978	1.19	1.26	0.1591	1.17	1.17
"	24.5	96	0.0673	0.72	1.38	0.4919	0.79	1.33	0.0972	0.68	1.27
2.0	25	84	0.0870	0.86	1.18	0.2134	1.31	1.09	0.1781	1.55	1.23
"	25.5	105	0.0805	1.18	1.18	0.2962	1.33	1.27	0.1880	1.61	1.18
"	24	108	0.0878	0.93	1.33	0.1863	0.98	1.27	0.1238	1.33	1.24
5.0	26	104	0.1045	1.05	1.30	0.4205	1.82	1.21	0.2760	1.71	1.18
"	25	118	0.0977	0.95	1.10	0.4697	1.95	1.02	0.3165	1.80	1.05
"	25.5	85	0.0945	1.29	1.25	0.4639	1.76	1.23	0.2328	1.30	1.14

comparison might be made. The fat contents of the yeasts incubated in the oxygenated solutions of glucose, fructose and sucrose were found to agree within limits of experimental error, but there was appreciably less fat in the yeast incubated in the maltose solution.

Table III. *Showing increase of fat and carbohydrate produced by incubating equal quantities of the same samples of yeast in 2 % solutions of two different sugars.*

Sugar 2 %	Temp. ° C.	Age of yeast. Hours	Original yeast		Incubated in oxygenated solution		Incubated in non- oxygenated solution	
			Fat g.	Carbo- hydrate g.	Fat g.	Carbo- hydrate g.	Fat g.	Carbo- hydrate g.
Glucose	25	103	0.0890	0.83	0.4818	0.84	0.2123	1.17
Fructose	"	—	—	—	0.4729	0.93	0.3007	1.12
Glucose	"	81	0.0785	1.53	0.4770	1.04	0.2096	1.04
Sucrose	"	—	—	—	0.4704	0.80	0.1996	0.93
Glucose	"	105	0.0805	1.18	0.3703	0.98	0.2074	1.22
Maltose	"	—	—	—	0.2962	1.33	0.1880	1.61

THE PART PLAYED BY OXYGEN IN PRODUCING AN INCREASE OF FAT.

In order to be sure that the effect of the oxygen was not merely the mechanical one of removing the carbon dioxide, such as that described by Slator [1921], we replaced the current of oxygen by one of hydrogen when incubating the yeast in a 5 % solution of glucose. The control in which no gas was passed gave figures similar to those obtained when a current of hydrogen was passed through the solution. It follows therefore that the effect of the oxygen is not a mechanical one but is specific to oxygen.

PART III. THE INFLUENCE OF PHOSPHATES.

The formation of hexosephosphate when sugars are fermented by yeast juice in the presence of phosphates was first established by Harden and Young [1905, 1908, 1909]; this compound appears to be fructose diphosphate and it is formed when either glucose, mannose or fructose undergoes fermentation. Whereas Harden and Young regard the formation of this compound as an essential reaction in the fermentation of sugar brought about by the living yeast cell, Neuberg, Levite and Schwenck regard its formation as a pathological phenomenon [1917]. Since its discovery a large amount of work has been carried out on the constitution and properties of this substance and evidence has been brought forward suggesting that the presence of a hexosephosphate may not be confined to yeast juice [Embden and Lacquer, 1921]. The probable presence of this substance in muscle [Embden and Lacquer, 1914, 1921] and the detection of an enzyme acting on it in ossifying cartilage [Robison, 1923] are also observations of great interest.

It is well known that the radicals of phosphoric acid and of the fatty acids are associated together in the lipins; the wide distribution of these compounds throughout both the animal and vegetable kingdoms has given rise to much speculation as to their function in the metabolism of the cell.

They have been regarded as the form in which fat is transported in the body, and evidence has been brought forward that in a lactating animal the phosphatide content of the blood in the mammary vein is less than that in the jugular vein [Meigs, Blatherwick and Cary, 1919, 1920]: from this the conclusion has been drawn that the phosphatides of the blood are utilised by the mammary gland to form milk fat. In spite however of the large number of investigations which have been carried out, the function of the lipins and the rôle they play in the life of the cell cannot be said to have been satisfactorily elucidated.

The fact that the hexose molecule and the fatty acid radicals are each found in association with the phosphate radical suggests that a more complete knowledge of the phosphorus metabolism of the cell may throw some light on the processes by which fat is manufactured from carbohydrate in the living organism. We endeavoured therefore to find out whether the formation of fat in the yeast cell was in any way influenced by the presence of phosphates in the solutions in which the yeast was incubated.

The first question investigated was whether the conditions under which the yeast was incubated could be so modified as to produce an increased storage of phosphate in the yeast cell. We therefore examined the effect of changes in the composition of the medium in which the yeast was incubated and determined the effect on the total phosphorus content of the yeast produced by the addition of sugar to the phosphate solution in which the yeast was incubated.

The method adopted was as follows: 12.5 g. of pressed yeast which had been well washed were incubated for 45 hours at 25° in a litre of a solution of sugar containing a mixture of 0.3962 % Na_2HPO_4 and 0.0286 % KH_2PO_4 , a mixture giving a p_{H} of about 7.8. The sugar to be investigated was then added in varying concentrations, the percentage of phosphate being kept constant throughout the series of experiments. At the end of 45 hours the yeast was filtered off, washed until the washings gave no reaction for phosphate and the total amount of phosphorus it contained determined by Neumann's method. An analysis of 12.5 g. of the original sample of yeast was also made so that the increase of phosphorus could be calculated. The fat and carbohydrate were estimated as previously described [Smedley MacLean, 1922] after hydrolysing the yeast by boiling for two hours with normal acid; the protein content of the yeast was also determined.

It was found that the increased amount of phosphate taken up by the yeast cell depended on the concentration of sugar in the solution in which the yeast had been incubated.

The effect of the three sugars, glucose, fructose and sucrose, was practically identical. The amount of phosphate taken up from the solution by the yeast cell varied with the concentration of sugar, increasing as the proportion of sugar in the medium rose from 0.5 to 5.0 %. The action of maltose also produced an increase in the phosphorus content of the yeast after incubation,

the increase being dependent on the strength of the maltose solution in which the yeast was incubated. The increase of phosphate in the yeast was however appreciably less when maltose was substituted for the other sugars mentioned, the difference being most marked in the stronger solutions.

The actual numbers obtained are shown in Table IV and the relation of the increase of phosphate to the concentration of the sugar solution is expressed by the curves shown in Figs. 5 and 6.

Table IV. *Showing amount of phosphorus in 12.5 g. of pressed yeast after being incubated for 45 hours in a litre of sugar solution containing 0.3962 % Na_2HPO_4 + 0.0286 % KH_2PO_4 .*

Amount of P in sample				Amount of P in sample			
Conc. of sugar %	(1) Original g.	(2) After incubation in oxygenated solution g.	(3) After incubation without oxygenation g.	Conc. of sugar %	(1) Original g.	(2) After incubation in oxygenated solution g.	(3) After incubation without oxygenation g.
Glucose:				Fructose:			
0.5	{ 0.0613	0.0687	0.0663	0.5	{ 0.0561	0.0777	0.0568
	{ 0.0617	0.0689	—		{ 0.0583	—	0.0586
1	{ 0.0618	0.0754	0.0734		{ 0.0679	0.0716	0.0656
	{ 0.0642	0.0764	0.0731		{ 0.0673	0.0713	0.0650
	{ 0.0602	0.0865	0.0893	1	{ 0.056	0.067	0.0697
	{ 0.0598	0.0850	0.0870		{ 0.057	0.062	—
2	{ 0.0611	0.0860	0.0786	2	{ 0.0682	0.1012	0.0900
	{ 0.0586	0.0870	—		{ 0.0718	0.1023	0.0921
	{ 0.0638	—	0.0740		{ 0.0598	0.0880	0.0747
	{ —	—	0.0730		{ 0.0566	0.0879	0.0757
4	{ 0.0650	0.1050	0.1024		{ 0.0611	0.0860	0.0761
	{ 0.0633	0.0989	0.0882		{ 0.0586	0.0846	0.0742
5	{ 0.0607	0.1021	0.1035				
	{ 0.0609	—	0.1124	5	{ 0.077	0.128	0.102
	{ 0.0580	0.1252	0.1158		{ 0.065	0.123	0.098
	{ 0.0586	0.1310	0.1140				
	{ 0.0694	0.1023	0.0962				
	{ 0.0694	0.1009	0.0919				
Maltose:				Sucrose:			
0.5	{ 0.0640	0.0566	0.0681	0.5	{ 0.0606	0.0668	0.0639
	{ 0.0637	0.0510	0.0653		{ 0.0578	0.0749	0.0634
1	{ 0.0634	0.0770	0.0602	1	{ 0.0620	0.0706	0.0632
	{ 0.0630	0.0706	0.0611		{ 0.0618	0.0717	0.0676
2	{ 0.0600	0.0776	0.0700	2	{ 0.0708	0.0934	0.0839
	{ 0.0594	—	0.0714		{ 0.0742	0.0965	0.0799
5	{ 0.0656	0.0949	0.0767	5	{ 0.0596	0.1102	0.0883
	{ 0.0684	—	0.0757		{ 0.0603	0.1105	0.0881
	{ 0.0709	0.0903	0.0772				
	{ 0.0679	0.0898	0.0780				

It is of course possible that the numbers obtained at the end of the experiment represent a balance; that for instance hexosephosphate is being continually formed and decomposed and that in the dilute sugar solutions the figures are low because there is insufficient sugar to reform the hexosephosphate which has been decomposed. The residual sugar was always determined at the end of each experiment; it was generally found that in the

0.5 % solutions all the sugar had been used up, but in the higher concentrations some sugar always remained.

The effect of oxygenation on the amount of phosphate taken up by the yeast cell was found to be quite marked. Oxygenation of the solution increased considerably the amount of phosphate stored by the yeast cell.

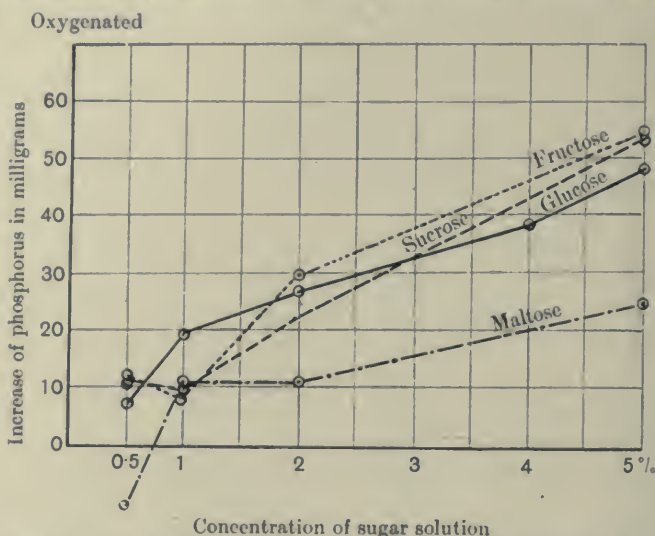


Fig. 5. Curves showing relation between concentration of sugar in solution and increase of phosphorus in yeast.

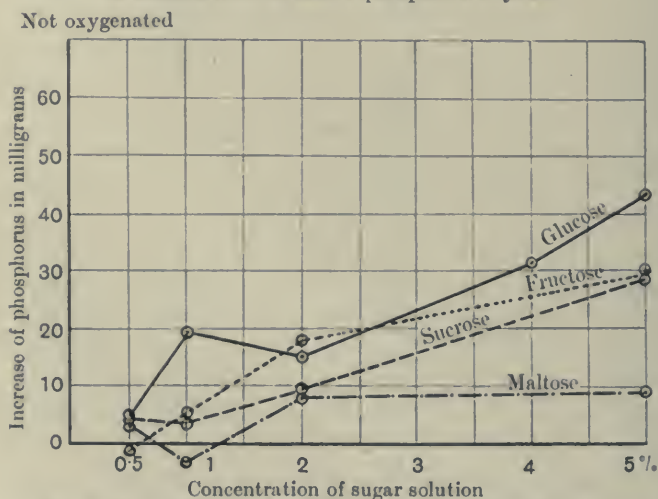


Fig. 6. Curves showing relation between concentration of sugar in solution and increase of phosphorus in yeast.

The above results indicate that phosphate is taken up by the yeast cell from the medium in which it is incubated, in association with sugar and strongly support Harden's view that hexosephosphate is a normal stage in the metabolism of the cell.

The second point investigated was whether the addition of phosphate to the sugar solution in which the yeast was incubated had any influence on the amount of fat or carbohydrate contained in the yeast.

The method by which this was determined was to incubate quantities of 12.5 g. of the same sample of yeast under precisely similar conditions in a litre of sterilised sugar solution with and without the addition of phosphate and to analyse the sample of yeast before and after it had been incubated. Four Winchester quart bottles were placed in the incubator at the same time; two contained a litre of the sugar solution which it was desired to investigate, and in each of the other two was placed a litre of the sugar solution to which the phosphate mixture had been added in the proportions above described.

The solution in one of each pair of bottles was oxygenated throughout the experiment so that the effect of oxygenating the solution could at the same time be determined.

INFLUENCE OF THE ADDITION OF PHOSPHATES ON THE FORMATION OF FAT.

The results of these experiments show that the presence of phosphates exerts a quite considerable effect in increasing the storage of fat if the solution in which the yeast is incubated is oxygenated during the experiment. If the solution be not so oxygenated the result is less marked, but in the majority of cases even when the medium is not oxygenated the yeast in the phosphate-containing solution has the higher fat content. Very dilute solutions of fructose were rather variable in their behaviour, but with stronger solutions the yeast always showed an increased fat content after incubation. The differences in the increase of fat produced by the addition of phosphates to the oxygenated solutions of the different sugars examined were of the same order; no differences which could be regarded as significant were observed between the effects of maltose, glucose, fructose or sucrose.

Analysis of the yeast samples before and after incubation clearly indicated that the addition of phosphates to the oxygenated sugar solutions produced an increased fat content of the yeast; we were however unable to establish a relation between the amount of increase of fat produced by the addition of phosphates to the oxygenated solution and the concentration of sugar in the medium.

INFLUENCE OF THE ADDITION OF PHOSPHATES ON THE CARBOHYDRATE CONTENT.

We have shown that oxygenation of the sugar solution in which the yeast is incubated on the whole tends to diminish the carbohydrate content of the yeast when compared with yeast grown in a similar solution without oxygenation; this effect is however not invariable and in most cases was found to be slight.

The effect of the addition of phosphates to the sugar solution was somewhat to increase the difference between the carbohydrate content of the

Table V. *Showing the effect of incubating yeast in solutions of 0.4 % alkaline phosphate with different concentrations of sugar. Influence on fat and carbohydrate content.*

Temp. °C.	Age of yeast. Hours	(1) Original sample of 12.5 g. pressed yeast			(2) After incubation in sugar solution oxygenated			(3) After incubation in sugar solution not oxygenated			(4) After incubation in sugar solution + phosphate solution oxygenated			(5) After incubation in sugar solution + phosphate solution not oxygenated		
		Fat g.	Carbo- hydrate g.	Protein g.	Fat g.	Carbo- hydrate g.	Protein g.	Fat g.	Carbo- hydrate g.	Protein g.	Fat μ.	Carbo- hydrate g.	Protein g.	Fat g.	Carbo- hydrate g.	Protein g.
Glucose:																
0.5	106	0.1040	1.02	1.23	0.1556	0.50	1.12	0.1318	0.66	1.16	0.2257	0.63	1.14	0.1198	0.75	1.07
1	85	0.0880	1.06	1.14	0.2432	0.71	1.15	0.1457	0.86	1.13	0.4335	0.70	1.16	0.1666	0.99	1.16
2	25	0.0905	1.37	1.15	0.3610	1.03	1.06	0.2220	1.27	1.09	0.4340	0.78	1.07	0.2580	1.02	1.09
25	94	0.0854	1.17	1.27	—	—	—	—	—	—	0.5389	0.98	1.19	0.2615	0.99	1.17
4	79	0.0772	0.89	1.27	0.2690	1.03	1.19	0.2470	1.17	1.20	0.4528	0.91	1.24	0.2391	1.02	1.22
5	87	0.1029	0.81	1.30	0.3215	1.18	1.27	0.2360	1.26	1.19	0.4522	0.87	1.22	0.2544	1.06	1.16
25.5	109	0.1102	1.02	1.26	—	—	—	—	—	—	0.9012	1.26	1.16	0.4542	1.02	1.11
Fructose:																
0.5	84	0.0800	1.46	1.19	0.3477	1.09	1.15	0.1183	1.08	1.15	0.3089	0.91	1.15	0.1089	1.13	1.15
25	88	0.0677	1.03	1.37	0.2380	0.77	1.26	0.1077	0.80	1.26	0.3231	0.87	1.24	0.1270	0.74	1.25
1	25.5	0.0925	1.17	1.26	0.4245	1.21	1.23	0.1414	1.08	1.14	0.3304	0.75	1.30	0.1464	0.92	1.26
2	83	0.0885	1.26	1.24	0.5465	1.08	1.14	0.2574	1.18	1.10	0.5495	1.05	1.18	0.1822	1.36	1.12
80	94	0.0848	1.45	1.18	0.3794	1.12	1.13	0.2395	1.34	1.17	0.5550	1.06	1.17	0.2534	1.19	1.14
25.5	94	0.0854	1.17	1.27	—	—	—	—	—	—	0.6167	1.06	1.18	0.2615	1.28	1.18
5	—	0.1046	0.82	1.43	0.5360	1.29	1.37	0.2254	1.10	1.36	0.8180	1.25	1.33	0.3680	1.63	1.35
Sucrose:																
0.5	82	0.0835	0.84	1.33	0.2516	0.73	1.15	0.0891	0.80	1.17	0.3334	0.72	1.17	0.1242	0.79	1.01
1	91	0.0706	0.77	1.37	0.3860	0.83	1.22	0.1457	0.82	1.23	0.3562	0.84	1.24	0.1277	0.83	—
2	84	0.0943	1.12	1.23	0.3980	0.90	1.16	0.2012	0.99	1.16	0.5441	0.94	1.13	0.2021	1.12	1.14
5	96	0.0888	1.10	1.28	0.6097	1.07	1.18	0.2398	1.06	1.12	0.7390	0.94	1.15	0.2669	1.17	1.15
Maltose:																
0.5	85	0.0867	1.02	1.32	0.2433	0.72	1.30	0.0935	0.72	1.21	0.3190	0.71	1.23	0.0818	0.46	1.25
1	96	0.0673	0.72	1.38	0.4919	0.79	1.33	0.0972	0.68	1.27	0.4960	0.81	1.29	0.1128	0.58	1.31
2	84	0.0870	0.86	1.18	0.2134	1.31	1.09	0.1781	1.55	1.23	0.3552	1.14	1.06	0.2083	1.22	1.16
24	108	0.0878	0.93	1.33	0.1863	0.98	1.27	0.1238	1.33	1.24	0.3454	0.70	1.26	0.1411	0.92	1.26
5	85	0.0945	1.29	1.25	0.4639	1.76	1.23	0.2328	1.30	1.14	0.5608	1.62	1.17	0.2335	1.48	1.03
25	96	0.1068	0.90	1.33	—	—	—	—	—	—	0.4907	1.48	1.25	0.2309	1.89	1.26

yeast incubated in the sugar solution with and without the passage of a current of oxygen. The carbohydrate content of the yeast incubated in the non-oxygenated solution was the greater and the addition of phosphate to the sugar solutions which were oxygenated during the period of incubation led to a distinct diminution in the carbohydrate content of the yeast.

INFLUENCE OF THE ADDITION OF PHOSPHATES ON THE PROTEIN CONTENT.

The differences in the protein content of the yeast before and after incubation in the sugar solutions to which phosphates had been added were very small; and considering the difficulties of carrying out these experiments without any loss of yeast during filtration, they cannot be regarded as outside the range of experimental error.

SUMMARY AND DISCUSSION OF RESULTS.

(1) When yeast was incubated in oxygenated water, part of the carbohydrate originally present disappeared and an increase of fat took place. The addition of propyl, butyl and *iso*-amyl alcohols in decimolar concentration exerted an inhibitory effect; decimolar solutions of the sodium salts of formic, propionic and butyric acids, glycol, glycerol and acetone behaved like water.

(2) When yeast was incubated in oxygenated 0.5 % solutions of ethyl alcohol or of the sodium salts of acetic, lactic or pyruvic acids, an effect was exerted on the fat content of the yeast similar to that produced by 0.5 % solution of glucose. Increasing the concentration of these substances in the solutions did not however lead to further increase of the fat content of the yeast, as in the case of the sugars.

A possible explanation is that these simpler molecules may be used to build up carbohydrate and only indirectly lead to the production of fat; this suggestion is supported by the observation that in certain cases where the yeast was incubated in oxygenated solutions of ethyl alcohol and of sodium acetate an increase in the total carbohydrate of the yeast cell was observed.

(3) When yeast was incubated under the conditions described in a solution of fructose, glucose or sucrose, at the end of 45 hours both fat and carbohydrate were found to have been stored up by the yeast cells. The amounts stored up depend on the concentration of sugar in the medium and are independent of the nature of the sugar; the rate of increase of the carbohydrate content diminishes as the concentration of the sugar in the medium rises; the rate of increase of the fat content also falls, but much more than that of the carbohydrate content, as the sugar concentration increases.

(4) Maltose differs markedly in its behaviour from the three sugars, glucose, fructose and sucrose. It is more potent in producing a storage of carbohydrate and rather less effective in building up fat.

The difference in the power of storing carbohydrate is so marked that it seems impossible that the maltose can be split into glucose before assimilation, and we must conclude that maltose is directly dealt with as such by the yeast

cell and there built up into reserve carbohydrate. It is interesting to recall that the recent work of Irvine [1923] points to the presence of a maltose unit in the glycogen molecule; this conclusion is in accordance with the behaviour of the yeast cell which stores carbohydrate more readily when fed with a solution of maltose than with one of glucose.

The storage of fat after yeast has been incubated in maltose solutions was rather less marked than with the other sugars; in 0.5 and 1 % oxygenated solutions as much fat was formed by the yeast as when it was incubated in the corresponding solutions of glucose, fructose or sucrose, but after incubation in a 2 or 5 % maltose solution less fat was formed. No increase in fat content corresponding with the increased carbohydrate content was observed.

(5) Oxygenation of the medium throughout the period of incubation made a very great difference to the amount of fat produced, but made little difference to the final carbohydrate content, generally producing a slight diminution in amount.

(6) The addition of phosphates to the oxygenated sugar solutions produced an increase in the amount of fat stored and a diminution in the amount of carbohydrate. If the solutions were not oxygenated, the increase of fat was small or absent.

(7) If about 0.4 % of alkali phosphates was added to solutions of the different sugars in which the samples of yeast were incubated, phosphate was taken up by the yeast cell in proportion to the concentration of sugar in the solution in which the yeast was incubated. The amount of phosphate was greater in the yeast incubated in the oxygenated solutions than in that incubated in solutions which were not oxygenated.

These results seem best explained by the hypothesis that carbohydrate storage in yeast takes place in two ways: (1) as glycogen or some other similar compound giving a reducing sugar on hydrolysis and (2) as a hexosephosphate which forms the first stage in the transformation of carbohydrate to fat.

The view that carbohydrate is absorbed and combined with phosphate forming a hexosephosphate is supported by the work of Harden and Young on the isolation of hexosephosphates from yeast juice and by the evidence now brought forward that the phosphate content of the yeast depends on the concentration of the sugar in the medium in which the yeast is incubated.

The second part of the hypothesis now put forward that the formation of a hexosephosphate may be regarded as the first stage in the transformation of carbohydrate to fat is supported by the fact that the addition of phosphates to the solution of sugar in which the yeast is incubated somewhat increases the fat content of the yeast if the solution be oxygenated during the period of incubation.

Although the proportion of oxygen is much less in the fat than in the carbohydrate molecule, the part played by the oxygen is evidently a very important one in the transformation of carbohydrate to fat. Oxygenation of

the sugar solution in which yeast is incubated produces a yeast with more than double the fat content of a yeast incubated in a sugar solution which has not been so oxygenated; and if additional phosphate be added to the medium the fat content is still further raised if the solution continues to be oxygenated.

The increase of fat stored up when phosphate is added to the oxygenated sugar solution in which the yeast is incubated seems to us to be best explained by assuming that oxidation of a hexosephosphate or of some material derived from it forms an essential stage in the story of the fat metabolism of yeast.

It is possible that simpler molecules such as those of pyruvic acid or aldehyde may be formed at a later stage, or it is possible that groups of this nature may be associated in some complex compound containing the phosphate radical. At present there is no evidence which throws light on this question and it remains only a matter of speculation.

We desire to express our thanks to Mr J. L. Baker and to Messrs Watney, Coombe, Reid and Co. for supplying us with yeast grown under approximately constant conditions and to acknowledge our indebtedness to the Food Investigation Board of the Department of Scientific and Industrial Research for giving us the opportunity of carrying out this work.

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XC. STUDIES IN BACTERIAL NUTRITION. I.

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IN approaching the study of bacterial nutrition there are two main lines of attack. First there is what may be called the analytical method, under which an attempt is made to separate a medium into its component substances or groups of chemically similar individuals, and then to determine which of these are essential to the life of the organism. Exceedingly little work has been done from this point of view, because no doubt of the lack of satisfactory methods for analysing proteins and their derivatives, and the difficulty of avoiding the introduction of bactericidal substances during chemical manipulation. The most recent work on these lines is that of Mueller [1922], who claims to have isolated a new amino acid, which he states to be one of the substances essential for the growth of the streptococcus.

The second method is the deductive one, which consists in following the changes occurring in the nitrogenous constituents of the medium during the growth of the organism, and thence attempting to elaborate a theory of its metabolism. A fair amount of attention has been devoted to this side of the question. Sears [1916] gives a comprehensive account of the progress so far made, and De Bord [1923] brings the summary up to date. The difficulty, however, of determining what relation the variation in amino acids, ammonia, and other end products, bears to the life history of the micro-organism, and in distinguishing between primary and secondary reactions in the medium, leaves the whole question as yet unsolved.

The object of the present research was to determine the growth substances for one organism in a given medium, by applying some of the chemical methods used for protein analysis. The medium was a pancreatic digest of caseinogen, first devised by Cole and Onslow [1916], but in the present case the whole pancreas finely minced was used instead of an extract; 100 g. of pancreas to 200 g. of caseinogen in a volume of 2 litres. This medium is used now in this laboratory as a stock medium. It gives with those organisms which will grow on a peptone-lemco medium, a luxuriant growth, which, however, is said rapidly to lose its viability. The use of a phospho-protein like caseinogen results in the finished medium being infinitely better buffered than most peptone media. The following table gives several chemical analyses of typical stock broths.

Analyses of stock broth 1 in 3.

% total nitrogen	% amino + ammonia N	% total solids	% ash	% ammonia N	% nitrogen unprecipitable by phospho- tungstic A
0.4110	0.2949	3.590	0.240	0.044	0.3162
0.3714	0.2786	3.044	0.218	0.032	0.2888
0.3267	0.2038	2.792	0.230	0.020	0.1880
0.3879	0.3139	3.370	0.244	0.035	0.2796
0.3671	0.3098	3.074	0.250	0.025	0.2367
0.3578	0.2571	3.070	0.218	0.024	0.2153

The amino acid and ammonia nitrogen were determined by formalin titration in alcoholic solution after the method of Foreman [1920]. This method gives a sharp end point, and is much more satisfactory for a liquid of this type which contains all stages of protein hydrolytic products, than either Sørensen's or Van Slyke's, both of which were tried in the first instance but subsequently discarded.

The organism decided upon was a streptococcus, by reason of its being intermediate in luxuriance between the easily growing coliform bacilli and staphylococcus, and the very delicate gonococcus and meningococcus. One thus has an organism which although reasonably easy to cultivate, seems to require certain specific substances for its growth, unlike bacilli of the coli type which can probably utilise many forms of nitrogen. The strain of streptococcus which was here used as the experimental organism was one which fermented only mannitol and saccharose, and was haemolytic, thus corresponding in Holman's classification to *Streptococcus haemolyticus* III. It was grown throughout on caseinogen digest medium, so that it should be fairly stabilised as regarded its requirements.

The first point proved was the heat stability of the growth substances contained in caseinogen broth.

Into each of 30, $3\frac{1}{2} \times \frac{3}{8}$ " tubes in a rack was measured 3 cc. of 1 in 3 stock broth, and after plugging with wool, the tubes were steamed for one hour. On 30 successive days the steaming was repeated, one tube being removed from the rack after each day's steaming; thus the tubes had from one to thirty hours' steaming. At the end of thirty days, when they were sown with a suspension of the streptococcus, no visible difference in growth was observed between the first and last tubes. Thus the growth substances are stable so far as a reasonable amount of steaming goes.

Autoclaving proved to be more destructive. A rack of 18 tubes of 1 in 3 stock broth was autoclaved for 20 minutes at 117° C. on successive days, one tube being removed each day, the procedure being similar to that employed in the steaming experiment. On sowing with the streptococcus, only the first eight tubes showed growth after 24 hours, but at the end of 48 hours, practically equal growth was present in all the tubes. Evidently prolonged autoclaving brings about some change in the broth which delays the growth of the streptococcus.

With regard to the efficiency of the growth substances it was found that growth of the streptococcus could be detected in an average sample of stock broth when diluted 1 in 96, which is equivalent to less than 0.1 of caseinogen.

The first line tried was the separation of caseinogen stock broth into two fractions by means of phosphotungstic acid, in an acid solution, according to Van Slyke's method [1912]. In preliminary experiments the effect of adding inorganic salts during chemical manipulation was very soon realised to be of importance. Rough experiments were carried out to determine their effect on growth, and the following results were obtained with substances liable to be introduced during the precipitation.

Sodium chloride. % NaCl added to stock broth:

	Control					
	*	0.5	1	2	3	4
Streptococcus	++++	++++	++++	++	++	+
Staphylococcus	++++	+	++	++	++	++
B. shiga	++++	++++	++++	++	±	-
B. coli	++++	++++	++++	++	+	+

* The stock broth used as control contained less NaCl than could be estimated by titration with AgNO_3 (i.e. less than 0.1 %).

Barium chloride. % BaCl_2 added to stock broth:

	Control					
	0.0	0.5	1	2	3	4
Streptococcus	++++	-	-	-	-	-
Staphylococcus	++++	++	+	+	-	-
B. shiga	++++	++++	++	++	±	-
B. coli	++++	+	±	±	-	-

Phosphotungstic acid. % phosphotungstic acid added to stock broth:

	Control					
	0.0	0.5	1	2	3	4
Streptococcus	++++	++++	++++	++	-	-
Staphylococcus	++++	++++	++	+	±	-
B. shiga	++++	++++	++	±	-	-
B. coli	++++	++++	++++	++	±	±

It will be noted in the case NaCl, that growth of all four organisms was impaired by a concentration of 2 %. This made it essential that HCl introduced into the broth should subsequently be removed by distillation *in vacuo* and not by neutralisation.

50 cc. of stock broth were treated with 18 cc. of conc. HCl and a solution of 15 g. phosphotungstic acid and the whole made up to 200 cc. The mixture was heated on a boiling water-bath until the precipitate nearly redissolved and then allowed to stand for 48 hours, at the end of which time the precipitate was filtered off through a hardened filter paper, and washed with 200 cc. of an ice-cold solution containing 2.5 % phosphotungstic acid, and 3.5 % HCl. In early experiments the removal of the phosphotungstic acid was effected by treatment with BaCl_2 in alkaline solution, but later the more convenient treatment with amyl alcohol-ether mixture (Van Slyke) was used, which avoids not only any considerable dilution, but also the introduction of toxic Ba salts. Both precipitate and filtrate were freed from phosphotungstic acid, evaporated to dryness *in vacuo* (temp. not exceeding 60°), and separately redissolved in distilled water, and adjusted to p_{H} 7.5. Growth was tested by placing the solutions in small tubes, plugging and sterilising (20 mins.

at 117° in the autoclave), and sowing with drops of suspensions of the streptococcus and other organisms from sterile dropping pipettes. The dilutions of all test solutions were so arranged that they were equivalent to the original 1 in 3 broth, or if this was inconvenient to 1 in 6 broth. Appropriate tubes of unaltered broth were always sown as controls. Growth was estimated by opacity and the following scale used.

- ++++ = Opacity equal to the control.
 +++ = Opacity equal to the control when the latter diluted with an equal volume of water.
 ± = Very slight or no evidence of growth, but growth obtained on subculturing on caseinogen digest.
 - = No growth on subculturing.
 ++ and +- = Intermediate degrees estimated by comparison with control and diluted control.

In neither of the two portions of broth prepared as above, nor in the two combined, was any growth whatever observed with the streptococcus, but with three other representative organisms tried the average results were as follows:

Organism	All solutions = broth 1 in 3:			
	Phosphotungstic precipitate	Phosphotungstic filtrate	Precipitate + filtrate	Control broth 1 in 3
Streptococcus	-	-	-	++++
Staphylococcus	+	++	+++	++++
B. shiga	+	+++	++++	++++
B. coli	+	+++	++++	++++

This served to show that for the last three organisms the growth elements in the broth could practically be reconstituted after chemical manipulation. Neither the precipitate nor the filtrate portion showed any growth inhibition when added to stock broth. It was evident that in carrying out the precipitation with phosphotungstic acid in the usual way some substance or substances, essential to the growth of the streptococcus, had been lost or rendered inactive. This missing growth factor was not to be found in the precipitate of humin, which is always formed when the phosphotungstic precipitate is extracted with amyl alcohol-ether mixture, for this proved to be quite inert as regarded growth of the organism, and it seemed likely that some chemical action accounted for the change in the broth.

Treatment with conc. HCl in the same proportion as used in the precipitation, and evaporation *in vacuo*, did not destroy the growth producing power of the broth. Under this treatment the only alteration which sometimes occurred was a change in the character of the growth, which became less agglutinated. Finally after many experiments varying the conditions of precipitation, it was found that impurity in the phosphotungstic acid accounted for the destruction of growth substances, and that when the crystals (British Drug Houses) were carefully repurified by treatment with conc. HCl and ether [Wu, 1920] the precipitation could be carried out by the usual method, and the broth practically reconstituted as regarded growth of the

streptococcus by adding the fractions together. In one experiment the growth in the reconstituted broth was quite as good as that in the control, but in general the results were as follows, some growth being lost.

	Precipitate (P)	Filtrate (F)	P + F	Control broth 1 in 3
Streptococcus	-	±*	+++	++++

* Apparently negative, but growth obtained on subculturing.

It is suggested that this slight growth in the filtrate portion was caused by the presence of a small amount of the precipitated basic amino acids, peptone bodies and ammonium compounds, the phosphotungstates of which are very slightly soluble, and that in the ideal experiment there would be two fractions of the broth neither of which would alone support growth of the streptococcus, but which would constitute a good medium when added together.

In one experiment in which stock broth was divided into two portions (the washings being included with the filtrate), the effect of adding increasing quantities of filtrate portion to decreasing quantities of precipitate portion was tried with the following interesting results.

	1	2	3	4	5	6	7	8	9	10	11	12
Filtrate cc.	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	Control
Precipitate cc.	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0	
Water cc.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
	-	+++	++++	++++	++++	++++	++++	+++	++	+	±	++++

The 6th tube ≡ broth 1 in 3

This seems to indicate that growth depends on the presence of a sufficient quantity of the precipitate portion, which, however, needs to be activated by the presence of a small amount of the filtrate portion.

It is interesting to note that the substance destroyed by the impure phosphotungstic acid, although essential to the growth of the streptococcus, does not affect the growth of the coliform bacilli or of the staphylococcus. Neither do the latter organisms seem to be at all exacting in their requirements, since their luxuriance depends apparently more upon the total nitrogen content of a given solution than upon the presence or absence of specific substances. There are definite indications on the other hand, that two substances at least are essential for the growth of the streptococcus; furthermore, these substances are reasonably stable and admit of chemical manipulation, so that the problem of their identity should be soluble by chemical means.

The precipitation by phosphotungstic acid promises to be of some use in the isolation of the growth substances of the streptococcus, and it is now being applied in conjunction with Dakin's butyl alcohol extraction in an attempt to fractionate the stock broth in a more detailed manner.

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XCI. THE FLUIDITY AND COAGULATION OF THE BLOOD.

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ONE of the landmarks in the history of the coagulation of the blood is the observation of Fredericq [1878] that heat coagulation occurs when the blood in the excised jugular of a horse is heated to 55–57°, after the subsidence of the corpuscles. As the fibrinogens of the laboratory prepared by the method of Hammarsten [1879] and of Schmidt [1892] coagulate at 56°, it is commonly believed that they are identical with the precursor of fibrin in the blood. This was not however accepted as conclusive by two prominent physiologists, Wooldridge [1885, 1893] and Michael Foster [1888], each pointing out that fibrin ferment (thrombin) could be injected into the circulation in massive amounts without causing thrombosis, while similar quantities added to solutions of the fibrinogens of the laboratory provoke clotting. An attempted explanation of this anomaly, in the terms of Howell's [1911] views on coagulation, was advanced by Gasser [1917], who suggested that thrombin, when intravenously injected, combines with antithrombin to form an inactive substance termed metathrombin, it being assumed, without any direct evidence, that the introduction of thrombin into the circulation causes the liver to secrete an excess of antithrombin. But Pickering and Hewitt [1922, 2] have shown that massive doses of thrombin can be injected into the circulation of animals deprived of hepatic activity without causing intravascular clotting and these authors have deduced evidence that antithrombins are *post-mortem* products. Thus the lack of coagulant action of massive doses of thrombin in circulating blood is at least suggestive of some essential difference in the condition or composition of the fibrinogens of the laboratory and the precursor of fibrin in the living blood.

The salts employed in the preparation of fibrinogen by Hammarsten's method cannot be regarded as without influence on the structure or condition of the latter material, as Howell [1914, 1] has found it is not possible by this method to obtain solutions of fibrinogen of uniform properties, part of the

¹ The theory of the fluidity and coagulation of the blood enunciated in this paper owes its origin and elaboration solely to J.W.P. but I am in entire agreement with it. The experimental work has been carried out in collaboration, the operative part by D. H. de S., the observations on clotting by both. D. H. de S.

protein being denaturated by repeated precipitation. The method of Schmidt [1892], which involves dilution with excess of distilled water, is not well calculated to preserve the surface conditions of colloidal complexes, and Mellanby [1909] has found that the fibrinogen prepared by this process is always contaminated with prothrombin. Only after prolonged adsorption by barium sulphate were Dale and Walpole [1916] able to disassociate prothrombin from fibrinogen. Accepting these data, we shall write of a fibrinogen-prothrombin complex in blood, rather than of two disunited substances fibrinogen and prothrombin.

Nolf [1921] has shown that fibrinogen dissolved in faintly alkaline isotonic solution and fibrinogen dissolved in the protein residues of the plasma behave differently with regard to precipitation by chloroform. This, he explains, as due to protection afforded by the stable colloids in the protein residue, which restore the condition of the prepared fibrinogen to that of the fibrinogen of circulating blood.

In view of the obvious differences of circulating and shed mammalian plasma, it appeared desirable to enquire into the condition of fibrinogen prior to bleeding, rather than subsequent to that act. To this end experiments were devised: (1) to study the effect of heating the blood in the heart to 56-63°, without exposure to the air; (2) to observe the results of heating intraventricular blood to 56-63°, after disturbance of its surface conditions; (3) to examine the coagulability of the fluid blood remaining in the heart, after the formation of mechanically produced intravascular clots and (4) to enquire into the effects on the coagulability of blood produced by heating it to temperatures less than the coagulation temperature of fibrinogen.

GENERAL TECHNIQUE.

Cats and rabbits were used for the experiments, the former were anaesthetised with A.C.E., the latter with ether. In the case of the cats the animals were pithed under the anaesthetic and artificial respiration was maintained. The great vessels round the heart were then tied by a mass ligature, the heart removed and immediately placed either in isotonic saline or in neutral olive oil. Under these conditions the hearts were heated without admission of air into their interiors for the periods and to the temperatures shown in the protocols.

Control experiments were made with a thermometer inserted, through the aorta, into the left ventricle and ligatured in position, so as to prevent the entrance of fluid from the bath into the cavities of the heart. These showed that four minutes sufficed for the intraventricular temperature to exceed 56°, when the heart was placed in a bath at 60° and that after seven minutes the temperature of the ventricular cavity was that of the bath.

The mechanically produced thrombi were obtained by the introduction of cotton threads into the cavities of the heart *in situ*. The contraction of the living heart muscle prevented haemorrhage from the punctures. By the term

"commencement" of coagulation is implied the first visible departure from fluidity, other than the formation of isolated filaments of minute diameter, by "completion" that coagulation has advanced so far that the vessel could be inverted without spilling.

Each experiment described is representative of a number yielding concordant results.

THE HEATING OF BLOOD IN EXCISED HEARTS.

Exp. 1. The heart of a cat was excised and placed immediately in an oil bath at 60°. The temperature of the bath fell to 56° and remained at that temperature for five minutes. The bath was raised to 60° and maintained at that temperature for twelve minutes. When removed from the bath, the heart showed a small clot in the right ventricle, with a considerable amount of fluid blood. This clotted on glass and some of it which remained in contact with the surface of the ventricle developed a large clot. The blood from the right ventricle also gave a precipitate when one-fifth saturated with ammonium sulphate and also when half saturated with sodium chloride. The small amount of blood in the left ventricle was completely fluid and subsequently clotted on glass.

Exp. 2. Cat. The clotting times of blood from carotid before excision of the heart were commencement 5' 30" and completion 6' 20". The heart was excised and placed in a normal saline bath at 62°. For the next eleven minutes the temperature of the bath varied between 60° and 63°, it was then kept at 63° for five minutes. All the blood in the right ventricle was fluid on shedding but subsequently developed small clots in contact with glass. These subsequently increased in size. The time of commencement of clotting was 4' 30", of completion of that process 7' 0", the times being in each case taken from the moment of shedding. The left ventricle was empty.

Exp. 3. Rabbit died under ether. Heart immediately excised and placed in oil at 65°. During the next seventeen minutes the temperature of the oil varied from 60–67°. It was then kept at 66° for six minutes. Examination of the right ventricle showed clots on the chordae tendinae and a considerable amount of fluid blood in which four small clots formed after it was shed on to glass. The residual fluid was not precipitated by either one-fifth saturation with ammonium sulphate or by half saturation with NaCl. The blood from the left ventricle behaved in a similar manner.

Exp. 4. Cat. The conditions were the same as in *Exp. 2*, except that the heart had stopped beating for eleven minutes before excision. After removal from the bath all blood was found to be fluid, except for some small clots in the left auricle. The fluid blood failed to clot.

Exp. 5. Cat. Heart excised 19 minutes after death from A.C.E. No heat applied. All blood fluid but subsequently completely clotted on glass. The coagulation times of blood from the carotid before death were: commence-

ment 7' 15" and completion 7' 55". Blood from the excised heart showed commencement of clotting 5' 10" and completion 8' 0".

A comparison of Exps. 4 and 5 shows that blood shed from a dead heart at room temperature clots but that the fluid taken from the cavities of a dead heart which has been heated from 56–63° behaves like blood which has been heated to a like degree either in a test tube or in a vein exposed to the air, *i.e.* the fibrinogen is destroyed. Blood taken from the cavities of a moribund heart (as in Exp. 3) which has been heated above the coagulation temperature of fibrinogen has the bulk of its fibrinogen destroyed. When however the freshly excised and still beating heart is immersed in a hot bath and is heated for 15–20 minutes to 56–60° fibrinogen remains in the blood contained in the ventricles. Such blood clots spontaneously when shed on to glass and is precipitated by one-fifth saturation with ammonium sulphate and by half saturation with sodium chloride (Exps. 1 and 2). Evidence is thus forthcoming that fibrinogen exists in blood, when unexposed to air or foreign surfaces, after heating above the coagulation temperature of the fibrinogens of the laboratory.

The next experiment illustrates the effect of sodium citrate on intracardiac blood heated to 56.5°.

Exp. 6. Cat. 3 kilo. A.C.E. pithed. Artificial respiration. 10 cc. of a 20 % solution of sodium citrate was injected into the jugular. The animal exhibited pronounced opisthotonus and its heart stopped. Blood withdrawn by aspiration from the right ventricle failed to clot, but clotted on re-calcification. The heart was excised and heated in a normal saline bath for 8½ minutes to 56–56.5°. On opening the ventricles the blood was found to be fluid. It did not clot on recalcification.

This experiment indicates that the intravascular injection of sodium citrate alters the condition of the fibrinogen moiety of the fibrinogen-prothrombin complex of the blood or of a protective colloid associated with fibrinogen in circulating blood.

A THEORY OF THE FLUIDITY AND CLOTTING OF THE BLOOD.

In introducing this theory a preliminary paragraph is perhaps not inopportune. Any hypothesis is merely a conceptual device for the correlation of experience and can lay no claim to finality. It is in this sense that the theory to follow is suggested. Hypotheses exhibit signs of senile decay when, to maintain them, it is necessary to postulate, as knowledge progresses, an increasing number of substances not actually observed or isolated. Such indications are evident in the current thrombin theories of coagulation.

The essential characteristics of the fibrinogen of the laboratory are:

- (1) It coagulates by heating to 56–57°.
- (2) It is clotted by moderate amounts of thrombin.
- (3) It is precipitated by one-fifth saturation with ammonium sulphate [McLean, 1920] and by half saturation with sodium chloride.

None of these features is exhibited by intravascular blood, provided its surface conditions have not been disturbed.

The disparity of this conclusion with the classic experiment of Fredericq on heating the "living test tube" to 56° becomes intelligible on consulting Fredericq's original memoir; for he found, almost invariably, a clot adjacent to the junction of the plasma and settled out corpuscles. Fibrin ferment (thrombin) was also present, although absent from the circulating blood of the horse. The colloids of this plasma had therefore progressed towards clotting.

The differences of the fibrinogen of the laboratory from that of undisturbed intravascular blood may be explicable in one of two ways:

(a) That fibrinogen does not exist in circulating blood, but that a precursor of it is present:

(b) That the fibrinogen in circulating blood is associated with a protective colloid, which renders it resistant to changes by the agencies named.

The following facts indicate that the latter interpretation is the more probable.

1. Fibrinogen is closely associated with prothrombin. On heating it can be resolved into two proteins exhibiting different coagulation temperatures [Huiskamp 1905; Iscovesco, 1906].

2. The protection against precipitation by chloroform resulting from dissolving laboratory fibrinogen in the protein residues of plasma [Nolf, 1921].

3. Both fibrinogen and prothrombin can be obtained from plasma after dilution with excess of distilled water, yet whole blood, even after contact with cut tissue, when so diluted and evaporated down to its original volume does not spontaneously clot [Pickering and Hewitt, 1921]. Lack of activation of prothrombin due to absence of "thrombokinase" cannot be pleaded as an explanation of these results, because the blood employed had been in contact with cut tissues, exhibited complete haemolysis following the dilution with distilled water and coagulated normally on glass when undiluted. The inhibition of clotting is however intelligible on the view that colloidal material is associated with the fibrinogen-prothrombin complex in circulating blood and the action of excess of distilled water may be regarded as stabilising such material.

It is suggested that the essential factors in the maintenance of the fluidity of circulating blood, in the clotting of shed blood and in the formation of intravascular clots are (1) a fibrinogen-prothrombin complex, (2) a protective colloid united to the fibrinogen-prothrombin complex in circulating blood and (3) salts of calcium.

No series of antibodies is postulated, as in the thrombin theories, but when the surface conditions of the blood are disturbed, either by shedding or by the introduction of disturbants *in vivo*, disunion of the protective colloid and the fibrinogen-prothrombin complex occurs. The inception of these changes is marked when blood slowly clots in oil by the appearance of a reversible gel [Pickering and Hewitt, 1921] and similar reversible material was found by

Pickering [1923] in the filaments formed immediately after bleeding in films of human blood exposed to moist air. From a comparison of the times of appearance of the reversible gels with the pre-clot changes in shed blood observed by Vines [1921, 1] and by Novy and De Kruif [1917], it would appear that the disunion of the protective colloid and the fibrinogen-prothrombin complex corresponds in time with the changes in the content of ionised calcium in shed blood, observed by the former worker, and with the development of toxicity recorded by the latter.

The next stage in the coagulation of the blood is the activation of prothrombin, yielding a *post-mortem* product thrombin. This may be effected by calcium ions [Howell, 1914, 1], or in blood in contact with damaged tissues by calcium ions and the hydrophile material called thrombokinase. On this view, both thrombin and thrombokinase would be regarded as accelerators of coagulation, rather than as initiators of that process. The prime factor in fluidity and clotting would thus be the surface conditions of the protective colloid, as when these remain undisturbed or if they are artificially stabilised then fluidity is preserved.

Working with fibrinogen purified by adsorption by barium sulphate and with prothrombin prepared by a modification of Mellanby's method, Dale and Walpole [1916] found that thrombokinase was necessary for the clotting of fibrinogen. Gratia [1918] found that calcium chloride, dissolved in distilled water, causes the lysis of platelets and it is generally recognised that disintegrated platelets yield thrombokinase. It is also generally accepted that prothrombin exists in circulating blood. Löwit [1892] found that the massive intravascular injection of calcium chloride fails to induce thrombosis and Pickering and Hewitt [1922, 2] obtained the same result with lethal intravenous injections of large amounts of calcium chloride, dissolved in distilled water, even after the intravascular injection of massive doses of thrombin into cats, deprived of hepatic activity. It would thus appear, accepting Dale and Walpole's results, that thrombokinase activates the prothrombin of the laboratory, but may not always invoke clotting under the conditions of circulating blood. This is intelligible as an example of protection *in vivo*, against the disturbant properties of the thrombokinase.

In the development of coagulation prothrombin is converted into thrombin and the union of fibrinogen and prothrombin is broken up. In this sense, the process of clotting is the lysis of a colloidal complex. Evidence is not wanting as to the similarity of protection against the precipitation or the coagulation of blood and protection against haemolysis. Numerous observers have shown that serum protects against haemolysis by several agencies, and Nolf [1921] has shown that the proteins of serum protect fibrinogen against precipitation by chloroform. The addition of egg-albumin to shed blood has long been known to delay clotting, and egg-albumin protects against haemolysis by eosin in the presence of sunlight [Schmidt and Norman, 1920], and also against haemolysis by saponin and by a heterologous serum [Pickering and Taylor, 1923].

The addition of an equal volume of 0.5 % solution of sucrose to blood inhibits coagulation. Eisler [1909] found that sucrose protects against specific haemolysis and observed that the addition of moderate amounts of electrolytes restores haemolytic action. Isotonic sucrose also inhibits the haemolytic action of saponin and sapotoxin [Furuhata, 1918]. "Peptone" inhibits the clotting of blood, and Schmidt and Norman [1920] have shown that "peptone" protects against haemolysis by eosin, in the presence of sunlight. Coagulability is restored to "peptonised" blood by the addition of moderate amounts of sodium chloride, by neutralisation with a weak acid and by the passage of carbon dioxide.

We have seen that the addition of moderate amounts of electrolytes restores haemolysis after protection by sucrose, and Pickering and Taylor [1923] have found that either acid or neutral egg-albumin affords less protection against haemolysis by saponin than does alkaline egg-albumin.

If the view is accepted that the problems of the fluidity and clotting of blood are comparable to those of the protection against, and the inception of, haemolysis, then they may ultimately be resolved into questions of the interfacial permeabilities of the fibrinogen-prothrombin complex of the plasma.

There remains for consideration a number of generally recognised phenomena in the fluidity and coagulation of blood, some of which have been neglected by the supporters of the thrombin theories, but all of which can be interpreted on the hypothesis just enunciated.

(1) *The fluidity of circulating blood.* It may be assumed that the surface conditions of the vascular wall are such as not to disturb those of the protective colloid of the plasma.

(2) *The condition of the plasma in the excised jugular of the horse.* Here partial clotting occurs with the formation of thrombin [Fredericq, 1878], but the bulk of the plasma remains fluid. This the thrombin fails to clot, in like manner to the failure of thrombin to clot circulating blood. This may be attributed to absence of disturbance of the protective colloid in those portions of the plasma in contact with the undamaged vascular wall.

(3) *The slowing of clotting on oiled and paraffined surfaces.* This is intelligible on the view that oil or paraffin only slowly causes surface change in the protective colloid of the plasma.

(4) *The fluidity of bird's blood in vitro.* This can be explained by regarding the protective colloid in bird's blood as more stable than that in mammalian blood. The observation of Rettger [1909] that bird's blood *in vitro* is clotted by a feather is explicable by disturbance of surface conditions by that agency. Coagulation by the rapid addition of tissue extract (thrombokinase) may be regarded as acting in a like manner.

(5) *The effect of re-precipitating and washing fibrinogens.* Howell [1916] found that the fibrinogens prepared from the oxalated plasmas of the bird and of the terrapin, by a single precipitation with sodium chloride, frequently

failed to clot on the addition of thrombin; but that the fibrinogens prepared by two or more precipitations were clotted by thrombin, provided the precipitates were sufficiently washed. This is intelligible on the view that the repetition of precipitation and washing disassociates the fibrinogen from the protective colloid.

(6) *The coagulant action of a thread.* The following type of experiment was devised to elucidate this.

Exp. 7. Cat. A.C.E. pithed. Artificial respiration for 20' before operating on the heart. Three cotton threads were introduced into the right and left ventricles, by passing threaded needles through the heart. The coagulation times of carotid blood, shed immediately before the introduction of the threads, were commencement of clotting 5' 50" and completion of that process 7' 0". A mass ligature was placed round the heart, which was excised while beating. Examination showed large clots surrounding the threads and some fluid blood. The latter on shedding on glass commenced to clot in 1' 0" and was completely clotted in 1' 20".

The preceding experiment indicates a sharp distinction between the residual fluid blood after the formation of mechanically produced thrombi and the blood remaining fluid after the formation of thrombi by the slow intravascular injection of tissue extract. In the former case the residual blood is hypercoagulable, in the latter it exhibits inhibition of clotting, the negative phase of Wooldridge. The hypercoagulable blood when shed closely resembles, in the speed of its clotting, blood shed after the intravascular injection of thrombin, while blood in the negative phase behaves like blood to which an anticoagulant has been added.

The production of a thrombus by a thread is intelligible as an example of the processes whereby a thread hastens the coagulation of shed blood. The electropositive ions in the fluid adjacent to the thread (the latter when wetted being electronegative) may well be regarded as disturbants in the first instance of the protective colloid and later of the fibrinogen-prothrombin complex. The fact that a considerable amount of coagulable blood was found associated with the thrombus indicates that any thrombin formed had no coagulant action on that portion of the blood whose surface conditions had not been disturbed. This may be attributed to the presence of undisturbed protective colloid, preventing the action of thrombin on the fibrinogen of the blood. In like manner may be explained the fact that the localised clot formed when an artery is ligatured does not lead to general thrombosis.

(7) *The action of organic intravascular coagulants.* Tissue extracts, some snake venoms and certain synthesised substances [Pickering, 1896], when rapidly injected into the circulation, produce intravascular clotting. This is intelligible on the view that the hydrophile qualities of these substances alter the surface conditions of the colloidal complexes of the plasma. Change in the distribution of electrolytes may also play a part.

(8) *The addition of a small quantity of water.* This hastens coagulation

in vitro and well may be a disturbant of any protective colloid present. It is noteworthy that Howell [1912] found that the addition of a small quantity of water to the shed blood of the terrapin induced clotting, but that addition of isotonic saline had no such effect.

(9) *The action of neutral salts on shed blood.* Relatively stable unions of these salts with either the protective colloid or the fibrinogen-prothrombin complex or both would account for the inhibition of clotting obtained. This view is concordant with the observation of Wooldridge [1893] that thrombin is absent from salted plasmas.

(10) *The action of oxalates and citrates*¹. This is probably dual in nature, a union of the salts with the protective colloid and/or with the fibrinogen-prothrombin complex, and also the removal of calcium, or, in the case of citrates, change in the ionisation of the calcium present. The former may stabilise the protective colloid, the latter may modify the subsequent activation of prothrombin. In the case of citrated blood or plasma changes in dispersion may also play a part.

Exp. 6 shows that the intravascular administration of sodium citrate disturbs the colloidal complexes of the plasma, for the fibrinogen of intraventricular citrated blood coagulates at the same temperature as does the fibrinogen of the laboratory.

The action of citrates *in vitro* is progressive. Freshly citrated blood is coagulated by addition of a non-retracted clot and by tissue extracts, but blood 18 to 23 hours after citration is not clotted by these agencies [Folley, 1918]. These facts are intelligible as due to the gradual adsorption of citrate by the protective colloid of the plasma, yielding, after prolonged action, a more stable protective complex than is at first formed. The fact that blood citrated for 18 to 23 hours can be clotted by the addition of small amounts of sodium chloride (Folley) is significant in connection with the clotting of "peptone" plasma by sodium chloride and in relation to the antagonistic action of certain concentrations of electrolytes to protection against haemolysis.

(11) *The inhibition of clotting by "peptone," nucleic acids and hirudin.* The anticoagulant action of "peptone" and of nucleic acids has been explained as due to excessive secretion, by the liver, of antithrombin or of antithrombogenic [Nolf, 1910]. But Pickering and Hewitt [1922, 1] have shown that inhibition of clotting occurs after the rapid intravascular injection of "peptone" into cats deprived of hepatic activity, and unpublished work by the same authors indicates that nucleic acids (freed from proteoses) exhibit a like effect under like conditions. It has also been shown that the addition of "peptone" to blood or plasma *in vitro* inhibits clotting, provided adequate precautions have been taken to preserve the surface conditions of the latter fluids [Pickering and Hewitt, 1922, 1].

On the view of coagulation enunciated, the anticoagulant action of "peptone" and of nucleic acids may be ascribed to the union of these sub-

¹ Work is in progress in this laboratory on citrates and fluorides.

stances with the protective colloid of the plasma, yielding a more stable protective complex than that existing in circulating blood. Several of the generally recognised reactions of peptonised blood point to this conclusion. It is clotted by dilution with water, by the passage of carbon dioxide and by filtration through a clay cell. Each of these agencies, as Wooldridge [1893] pointed out, tends to dissolution. The disruption of a relatively stable union of a protective colloid and "peptone," under the conditions of shed blood, may be expected to cause clotting. "Peptonised" blood ultimately clots, when exposed to the air in glass vessels. This is intelligible as the gradual resolution, under the stress of disturbed surface conditions, of the union of the "peptone" with the protective colloid of the plasma. The addition of tissue extract rapidly coagulates "peptonised" blood. This may be attributed to lysis by the hydrophile material of the tissue detritus. On the other hand, thrombin fails to clot "peptonised" blood [Wooldridge, 1893]. On the view advocated, the difference between "peptonised" and normal shed blood is that in the former the protective colloid is stabilised by union with "peptone," in the latter it has undergone surface change. Thrombin only acts as a coagulant after surface change has disunited the union of protective colloid and the fibrinogen-prothrombin complex of the plasma. In "peptonised" blood this union is preserved and thrombin has consequently no action.

Hirudin exhibits its anticoagulant action after injection *in vivo* and admixture *in vitro* and its union with the protective colloid of the plasma is more stable and less toxic than are the unions of plasma with "peptone" or nucleic acids. Its action and its neutralisation by tissue extracts are explicable on the views stated. At a later stage in coagulation, hirudin also apparently combines with thrombin [Vines, 1921, 2]. The fact that "peptone" has no anticoagulant action on blood shed in the absence of precautions to preserve its surface conditions points to the union of the "peptone" with the unchanged protective colloid of the plasma. Hirudin, however, may unite with the colloids of the plasma, after disturbance of their surface conditions. Nucleic acids occupy an intermediate position, for they exhibit anticoagulant action after intravascular injection, after addition to blood shed on paraffined surfaces and also when blood is shed on glass. The degree of anticoagulant action is greatest when nucleic acids are injected *in vivo*, least when added to blood shed on glass, the action on blood shed on paraffined surfaces occupying an intermediate position.

(12) *The negative phase of coagulation.* No satisfactory explanation is afforded by the thrombin theories. A negative phase in the coagulation of bird's blood *in vitro* has been produced by Pickering and Hewitt [1921] by the slow addition of tissue extract, drop by drop, to that fluid. In the paper recording this fact attention was directed to the negative phase or tolerance exhibited in the precipitation of colloids by the slow addition of electrolytes, in precipitation of gelatin by the slow addition of alcohol, in the fractional neutralisation of certain toxins by antibodies (known as the Dansyz [1902]

reaction), and in the fractional neutralisation of the toxic qualities of arsenious acid by ferric hydroxide. It was submitted that these phenomena are of one class and are explicable in the same manner as physico-chemical processes.

On the view that a protective colloid is associated with the fibrinogen-prothrombin complex of the plasma, the *rapid* addition of tissue extract *in vivo* or *in vitro*, possibly by creating inequality of electric charge, tends to dissolution of the protective colloid and to coagulation; the *slow* addition of the same substance tends to uniformity of charge and to the formation of unions with the protective colloid, which unions (of protective colloid and tissue extract) exhibit physical resistance to further disturbance by the tissue extract.

In this connection, the reversed or negative action of "peptone" on the coagulability of blood demands notice. "Peptone" when introduced into the circulation slowly, drop by drop, not only does not delay clotting but produces partial or complete immunity against the anticoagulant action of subsequent injections of "peptone." This occurs both in intact animals and in those deprived of hepatic activity. This immunity reaction is intelligible on the view that the slow introduction of the "peptone" promotes unions with protective colloid of the plasma, which unions exhibit physical resistance against further unions with "peptone."

(13) *The action of silica sols.* These afford a marked contrast to the action of tissue extracts. Gye and Purdy [1922] found that lethal intravenous injections produced extensive thrombosis, that fractional injections induced hypercoagulability and that their addition to shed blood did not alter the speed of clotting. If we accept the conclusion of these observers that the lethal injections damaged the vascular wall, then the thrombosis is explicable. For damage to the vascular wall alters those surface conditions, which are essential to the preservation of the stability of the protective colloid of the plasma. The absence of a negative phase and the lack of coagulant action *in vitro* indicate that sols of silica neither unite with nor directly disintegrate either the protective colloid or the fibrinogen-prothrombin complex.

(14) *The influence of temperature.* That cooling retards clotting and heating hastens that process are generally accepted, but no unanimity exists among observers as to the variations in the speed of spontaneous clotting arising from increasing temperatures. This may be accounted for by the wide divergences in the methods adopted to determine coagulation time, and by the varying degrees of contact of the blood with damaged tissues. In the experiments to be described attempts were made to eliminate or minimise this.

Exp. 8. Blood was shed from the carotid of a pithed cat through an evenly paraffined cannula into wide mouthed glass vessels each of the same calibre. The vessels were submerged above the level of the blood in them in water-baths, raised to the desired temperatures. Evaporation was minimised by covering the vessels with moist filter papers. As it is known that hypercoagulability may result from successive haemorrhages, observations at any one temperature were repeated at different stages of the bleeding.

The graph (Fig. 1) shows the speeds of clotting corresponding to temperatures ranging from 33–57°. The points on the upper line of the graph represent the times of completion of coagulation, those on the lower line show the times of commencement of that process.

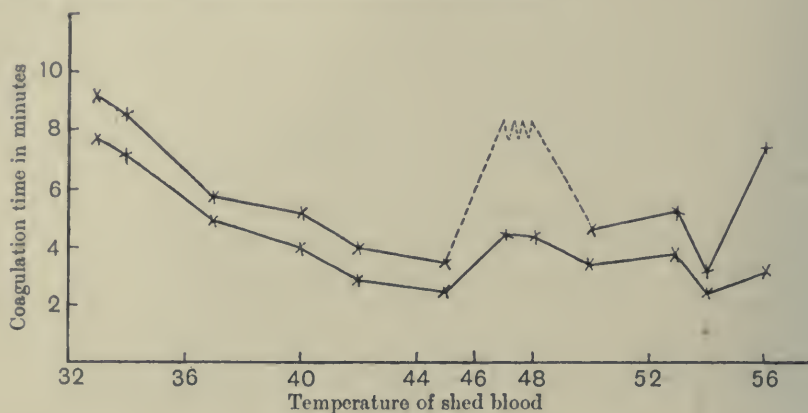


Fig. 1.

Exp. 9. Human blood was obtained by bleeding from serial punctures in the fingers, after cleaning with chloroform and ether and coating the skin with paraffin wax. The paraffined surface was cleansed after each bleeding. The points in the graph (Fig. 2) have the same significance as in Fig. 1. In this experiment contact with damaged tissue is not eliminated, but contact with clotted blood and foreign material is minimised.

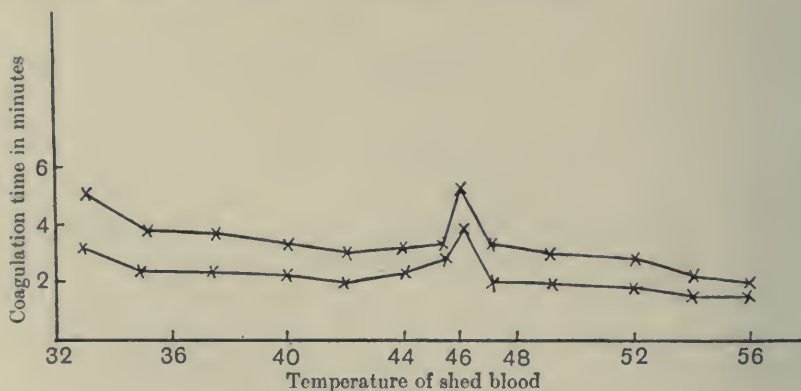


Fig. 2.

Irregular results were obtained in other experiments in which the same puncture was repeatedly used, when bleeding was not on paraffined surfaces and when pressure was exerted to squeeze out the blood.

The following is indicated by Fig. 1: (a) In the absence of contact with cut tissues and at temperatures ranging from 33 to 42°, the speed of spontaneous coagulation of the blood of the cat shed on glass increases regularly

with rises of temperature. (b) From 42 to 45° increase of temperature increases the speed of clotting, but the acceleration is not so marked as with increases at lower ranges of temperature. (c) At 47° marked slowing occurs in the commencement of coagulation and the end point of clotting is not well marked. The type of clot underwent profound change on heating to 47–48°. At lower and higher temperatures (below 56°) clotting was completed by the formation of a continuous gel, so that the vessel could be inverted without spilling blood. At 47–48° a succession of small clots was formed and the phase of a continuous gel was absent. The small clots contracted and progressive lysis of these clots was observed. It is significant that these changes in coagulability and the appearance of lysis of clots occur at a temperature which corresponds with that at which death and the onset of heat rigor is manifested in mammalian muscle [Brodie and Richardson, 1899]. In this connection, the views of Nolf [1909] on the supposed metabolic importance of the lysis of clots and the assumed secretion of antifibrinolysin by the liver may be recalled.

The second graph (Fig. 2) illustrates the retardation of the speed of clotting at 46° obtained with human blood which had passed through punctured tissue. With this blood the end point of coagulation was marked by the formation of a continuous gel.

These results are concordant with the view that physical or chemical changes or both occur either in the protective colloid or in the fibrinogen-prothrombin complex at the time of the death of the blood. Whether the lysis of the complexes of plasma is due to death by heat future experiment must determine. From a number of preliminary experiments on heating pithed animals to 47–49° it is evident that coagulability is not only modified but that intravascular clots may be produced. It is proposed to return to this subject later.

SUMMARY.

(1) Blood in the freshly excised heart, with its cavities unexposed to air, can be heated for 15 to 20 minutes to 56–60° without the destruction of fibrinogen. Such blood coagulates when shed, is precipitated by one-fifth saturation with ammonium sulphate and by half saturation with sodium chloride.

(2) Blood in a dead heart when heated under like conditions has its fibrinogen destroyed. Blood in a moribund heart has its fibrinogen partially destroyed.

(3) If intraventricular blood is citrated and recalcified *in vivo*, its fibrinogen is completely destroyed on heating, without exposure to the air, to 56–56.5° for 8½ minutes.

(4) A theory of the fluidity and the coagulation of the blood is enunciated. The observations recorded and the generally recognised phenomena of fluidity and clotting are correlated by this hypothesis.

(5) Attention is directed to the similarity of phenomena in protection against the spontaneous coagulation of blood and those of protection against haemolysis.

(6) The fluid blood adjacent to mechanically produced thrombi is hypercoagulable. Attention is directed to the contrast in the condition of this blood with that of the fluid blood associated with the thrombi formed in the negative phase of clotting. Reasons are given why the thrombus produced by ligaturing a living artery does not produce general thrombosis.

(7) Observations are recorded on the influence of heat on the coagulability of the shed blood of the cat, after precautions had been taken to prevent contamination with tissue detritus. The influence of heat on the coagulability of human blood after contact with the surfaces of a puncture, is also described. Both these bloods exhibit marked changes in coagulability on heating to 46–48°, *i.e.* at the temperature of death and the onset of heat rigor in muscle.

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XCII. AN IODOMETRIC METHOD FOR THE ESTIMATION OF NITROGEN IN OSAZONES.

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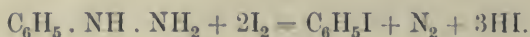
It is well known that those compounds containing nitrogen atoms directly linked together do not respond quantitatively to the Kjeldahl process, and for the estimation of nitrogen in such cases resort must be had to Dumas' method. Now although this method leaves nothing to be desired on the score of accuracy, it possesses certain disadvantages. Thus, apart from the question of time and attention required, it not infrequently happens that the quantity of material at disposal does not amount to more than a few decigrams. Among those compounds to which these remarks apply may be mentioned phenylhydrazine and its derivatives, and the method to be described was devised more especially for estimating nitrogen in osazones.

Grimaldi [1903] has suggested a method for the estimation of hydroxylamine in oximes, and phenylhydrazine in hydrazones and osazones by heating these compounds in a flask having a long narrow neck with a known volume of standard hydrochloric acid in excess in a boiling water-bath for one hour. The aldehyde or the ketone is regenerated and the liberated hydroxylamine or phenylhydrazine forms the hydrochloride. The excess of the uncombined acid is then titrated with $N/100$ alkali. From the amount of acid neutralised the hydroxylamine or phenylhydrazine may be calculated. This method appeared to be a convenient one for the estimation of nitrogen in osazones of sugars. The original communication not being available for consultation I am without details of the author's procedure. Some preliminary experiments were therefore carried out to determine the concentration of acid necessary for decomposing the osazones. It was found that $N/10$ – $N/100$ hydrochloric acid did not act on glucosazone under these conditions, but N hydrochloric acid brought about the expected decomposition.

The method entailed the titration of N acid with $N/100$ alkali. It was therefore useless for delicate work and had to be abandoned.

The reaction still seemed interesting and it was investigated in another way. Instead of titrating the excess of the acid after decomposing the osazone under the above conditions it appeared possible to estimate the phenylhydrazine formed during the reaction by the iodometric method described in the

previous paper by Ling and Nanji [1921]. This method of estimating phenylhydrazine depends on the fact that when a solution of phenylhydrazine is added to an excess of a standard solution of iodine it is decomposed according to the equation:



The reaction is an extremely sensitive one and has proved very useful in studying the decomposition of osazones with hydrochloric acid. The method employed was as follows.

A known weight of the osazone was heated with a known volume of *N* hydrochloric acid in a boiling water-bath for one hour. The reaction mixture was then neutralised with an equivalent amount of standard alkali, acidified with a drop of dilute acetic acid, and then made alkaline with an excess of pure sodium bicarbonate solution. The mixture was then washed into an excess of a known volume of standard iodine solution and the excess of iodine titrated with sodium thiosulphate, the difference being equivalent to the phenylhydrazine formed in the reaction mixture.

It was found by studying the above reaction between osazones and hydrochloric acid by this means that the decomposition did not proceed quantitatively, but it proceeded to an extent which was determined by the relative amounts of nitrogen present in the sample and the volume of the hydrochloric acid used. When the amount of hydrochloric acid was kept constant and the amount of nitrogen increased the percentage of the osazone decomposed decreased steadily. As a result of a large number of titrations it was observed that so long as the ratio of the nitrogen to the hydrochloric acid used was fixed, the percentage of the osazone decomposed was constant. Accordingly a curve was obtained by keeping the amount of hydrochloric acid constant and varying the amount of nitrogen present. When this curve is applied to different sugar osazones very concordant results are obtained. This has been made the basis of an empirical method for the estimation of nitrogen in sugar osazones, and it gives trustworthy and accurate results as will be seen by the following experiments, so long as the estimations are carried out under definite conditions.

The method is as follows:

A convenient weight of the osazone which may vary from 5–60 mg. is accurately weighed out in a Freudrich flask. To it is then added 10 cc. of *N* hydrochloric acid, and the mixture heated in a boiling water-bath for one hour. The reaction mixture is washed into a beaker and to it is added 10 cc. of *N* sodium hydroxide. It is then acidified with a drop of dilute acetic acid and the solution made alkaline with an excess of pure sodium bicarbonate solution. The alkaline solution is then washed into an Erlenmeyer flask containing an excess of a known volume of standard *N*/50 iodine solution. The excess of the latter is then determined by titrating with a standard solution of sodium thiosulphate. The number of cc. of *N*/50 iodine used on being

referred to the following table corresponds to the amount of nitrogen present in the sample taken, from which the percentage could be calculated.

cc. of N/50 iodine used	mg. of nitrogen	cc. of N/50 iodine used	mg. of nitrogen	cc. of N/50 iodine used	mg. of nitrogen
4.7	0.782	14.0	3.371	22.0	6.757
5.0	0.839	15.0	3.628	23.0	6.980
6.0	1.029	16.0	3.885	24.0	7.203
7.0	1.219	17.0	4.142	25.0	7.426
8.0	1.409	17.3	4.220	25.7	7.582
9.0	1.599	18.0	4.428	26.0	7.679
9.3	1.656	19.0	4.723	27.0	8.002
10.0	1.958	19.1	4.752	28.0	8.325
11.0	2.389	20.0	5.972	28.6	8.518
12.0	2.820	20.3	6.378	29.0	8.754
12.2	2.906	21.0	6.534	29.5	9.049
13.0	3.114	—	—	—	—

The following are some of the results obtained with different osazones. They represent the mean values of a number of estimations with quantities of the osazone employed varying from 5-60 mg.

	N	Found	15.52 %	Calculated	15.64 %
Glucosazone	N	..	10.56	..	10.76
Maltosazone	N	..	10.65	..	10.76
Isomaltosazone	N	..	8.14	..	8.21
α -Glucosido-isomaltosazone	N	

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XCIII. STUDIES ON CARBOHYDRATE METABOLISM.

III. ON THE FORMATION OF AN ANTI-DIABETIC HORMONE BY THE ACTION OF A BACILLUS. (PRELIMINARY COMMUNICATION.)

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(Received August 28th, 1923.)

WORKING with samples of commercial yeast, it was found [Winter and Smith, 1923] that an extract could be prepared which had an effect similar to insulin when tested on normal animals and diabetic persons. Later it was found [Hutchinson, Smith and Winter, 1923] that extracts from different samples of commercial yeast differed widely in their activity. It was improbable that these differences were due to variations in the nutritional state of the yeast, and it seemed possible that the different commercial samples of yeast might contain quantities of organisms, which are responsible for the whole of the insulin-like activity. It has been found that certain yeasts in pure culture give appreciable quantities of an anti-diabetic hormone, while others are quite inactive in this respect. The possibility was not excluded, however, that there might be in commercial yeast some micro-organism, which was not a yeast, but which was capable of forming an insulin-like substance.

The following is an account of some work directed to test this possibility. The anti-diabetic hormone will be referred to as glucokinin, though it has been pointed out [Hutchinson, Smith and Winter, 1923] that the substance so named and described by Collip may be different from that which we propose to discuss.

A portion of a sample of yeast was extracted with alcohol and the activity of the extract tested on rabbits. The blood sugar determinations were made by Bang's old method. The effect on the blood sugar was to cause a moderate fall, as will be seen from the following experiment.

Rabbit weight 1.8 kg.

Time	Blood sugar
	o/ o
10-00	.14
10-10	Injected .06 mg./g.
12-00	
3-00	.10
5-30	.12
	.14

A portion of the same yeast was plated out on nutrient agar and a pure culture of a bacillus obtained. This was sown into 100 cc. peptone water containing 1 % glucose, and incubated at 37°. Rapid growth took place, and after two days alcohol was added to make the mixture 75 %. The fluid was placed in the ice-chest over-night, filtered, and concentrated to 20 cc. It was then made up to 80 % with alcohol and allowed to stand in the ice-chest for 12 hours. The supernatant fluid was then made up to 95 % with alcohol, when an appreciable precipitate was formed. Precipitation was complete in 24 hours at - 7°. The precipitate was washed with absolute alcohol, and dry ether, and dried. This powder was tested on rabbits, the standard injection being given (-06 mg./g. body weight). A marked fall in the blood sugar occurred. It appeared probable therefore that an appreciable amount of glucokinase had been formed by the action of the bacillus. Control experiments were performed in which the sterile medium was extracted with alcohol, and the resultant powder injected into a rabbit. No fall in the blood sugar occurred, though the yield of extract was similar in quantity.

Rabbit weight 1.9 kg.

Time	Blood sugar %
10-15	-12
10-30	Injected (bacillus extract)
11-45	-09
1-00	-08
3-30	-12

Rabbit weight 2 kg.

Time	Blood sugar %
10-00	-10
10-15	Injected (control)
11-30	-10
12-50	-11
2-30	-10

In the previous experiments in which the bacillus was grown in the glucose peptone water the reaction at the end of the experiment was found to be markedly acid. Since it is well known that phosphates are of importance in the metabolism of carbohydrate, the addition of phosphate was employed, both for this reason, and for the purpose of controlling the reaction of the fluid. The bacillus was sown in the following media.

I. 200 cc. peptone water containing 1 % glucose and 1 % Na_2HPO_4 .

II. The same as above, but with NaH_2PO_4 in place of the di-sodium salt.

Growth was allowed to continue for four days when the extracts were made as before. The activity was greater in the slightly alkaline medium, the following being results obtained from tests on rabbits.

Rabbit weight 2 kg. Na_2HPO_4 .

Time	Blood sugar %
11.00	-10
11.15	Injected
12.20	-08
3.00	-06
4.45	-05
6.00	-04
6.10	Convulsed
6.20	Glucose injected
6.45	Animal eating
9.00	-11

Rabbit weight 1.7 kg. NaH_2PO_4 .

Time	Blood sugar %
10.00	-09
10.15	Injected
11.30	-07
1.00	-09
3.00	-10

In another experiment in which tri-sodium phosphate was used, no growth occurred. In the above experiment in order to make the conditions com-

parable the reaction was adjusted in each case to p_{H} 5 before concentration *in vacuo*. Though it is uncertain whether the substance produced by the bacillus has similar properties to insulin, the known stability of insulin to acid suggested that concentration should preferably be carried out in acid solution. We have since found, however, that concentration can be carried out in a slightly alkaline medium. Some further experiments have been performed with a view to comparing the yield of active principle obtained under different conditions. The strength of glucose in the peptone water was varied from 1 to 2 %. No appreciable variation in the activity was found. In another experiment the bacillus was sown in peptone water without glucose in the presence of 1 % Na_2HPO_4 . Growth was allowed to continue for four days. Moderate activity resulted. The following shows the results of experiments with batches grown with and without glucose.

With glucose			Without glucose		
Rabbit weight 2 kg.			Rabbit weight 2.5 kg.		
Time		Blood sugar %	Time		Blood sugar %
11.00		.10	10.00		.11
11.15	Injected		10.15	Injected	
12.20		.08	11.30		.08
2.30		.06	12.30		.07
4.30		.05	2.00		.09
6.00		.04	3.30		.10
6.10	Convulsions				
6.20	Glucose				
6.35	Animal eating				
8.00		.11			

To determine the effect of aeration on the production of glucokinase, the bacillus was sown in peptone water containing 1 % glucose and di-sodium phosphate. The mixture was incubated at 37°, a stream of sterile air being drawn through the mixture for two days. Abundant growth took place. At the end of this period the action was stopped, and the usual alcoholic extract made. On testing this however on rabbits it was found that the activity was much diminished.

In view of the considerable quantities of alcohol necessary, experiments were carried out in which the medium in which the bacillus had grown was concentrated *in vacuo* without preliminary treatment with alcohol. 250 cc. of medium were concentrated to 25 cc. and made up to 80 % with alcohol. The precipitate formed was very large. When this had settled the supernatant fluid was made up to 95 % alcohol as before. The resulting precipitate contained only a small amount of active principle. Presumably the first precipitation in a large quantity of alcohol tends to remove interfering substances without causing appreciable loss of glucokinase, but when these substances are precipitated in a small quantity of 80 % alcohol the glucokinase is largely carried down with them. Further experiments are being made in order to remove these substances in the first stage by another method without the use of alcohol.

DISCUSSION.

That the convulsions caused by the injection of an extract of the medium in which the bacillus had been grown were not due to a toxic effect was shown by the fact that the animals invariably recovered as a result of injection of glucose. The effect therefore would seem to be similar to that produced by insulin or an extract of yeast with consequent hypoglycaemic convulsions. The active principle obtained by the action of the bacillus would appear to be similar to that obtained from yeast since in both cases the same delayed action is noticed. Convulsions usually occurred six hours after injection in the case of an active extract. Only one injection was necessary to cause convulsions in the case of such an extract.

The order of the yield obtained is as follows. 200 cc. of medium in which growth had taken place for four days yielded 0.6 g. of powder of which 120 mg. caused convulsions in a rabbit of 2 kilos.

We have not yet identified the organism used in these experiments. It is a short coli-form bacillus, producing acid and gas from glucose or lactose in 12 hours. It does not liquefy gelatin. Similar experiments carried out with a strain of *B. coli* (for this we are indebted to Miss M. Stephenson) showed that no active principle was formed. Experiments are in progress to determine whether other bacilli of this group can form glucokinin.

SUMMARY.

1. An insulin-like substance is formed by the action of a coli-form bacillus.
2. Injection of the extract into rabbits causes hypoglycaemic convulsions which are relieved by glucose.

We wish to thank the Department of Scientific and Industrial Research (W.S.) and the Medical Research Council (L.B.W.) for personal grants held during the course of this work.

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XCIV. THE EVALUATION OF THE PURITY OF VARIOUS ORGANIC PRODUCTS BY THE DICHROMATE METHOD.

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(Received August 21st, 1923.)

THE writer has previously described [1914] a method for the volumetric estimation of carbon, the essence of which is oxidation by a mixture of phosphoric acid and potassium dichromate and the measurement of the CO_2 produced. It was shown that where methyl groups are present the substance is not oxidised to carbon dioxide alone but to a mixture of carbon dioxide and acetic acid; for example, lactic acid, alanine and ethyl alcohol each yield one molecule of acetic acid per molecule of substance taken; that is to say each methyl group gives rise to one molecule of acetic acid. The method proved of service in estimating the amount of such substances as mannitol and glycerol or, in the case of a mixture, of the residual oxidisable material after a fermentation. It is possible with a few cc. of solution to estimate how much material remains after fermentation, and so on.

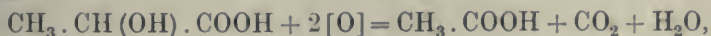
At the time of publishing this method the writer was unaware of Martin's method [1904] for the estimation of alcohol. Martin's method is now probably fairly generally employed in industry; certainly in France. It is exceedingly accurate and is applicable to very dilute solutions of alcohol. Martin distils the alcoholic solution into a mixture of equal parts of *N* potassium dichromate and sulphuric acid; the steam keeps the mixture hot and the oxidation is immediate, the alcohol being oxidised to acetic acid (1 cc. *N* dichromate = 0.0115 g. alcohol). The measurement of the reduction of standard dichromate solution is made by a solution of iron of about a fifth the strength of the dichromate, which increases the speed and accuracy with which one can work. The method is clearly much simpler than my own but is without some of its advantages; one cannot for instance apply it to the oxidation of a substance without knowing whether it is oxidised to acetic acid or to carbon dioxide or both. Provided however we know what are the products of oxidation, we could probably employ the dichromate method for estimating many substances; and its application to the estimation of impurities in non-oxidisable substances seems obvious.

In the second place, by a combination of Martin's method with my own one can obtain two constants which permit the estimation of the proportions of many substances, *i.e.* (1) the dichromate method, measuring the dichromate

reduced; (2) the dichromate method measuring the CO_2 produced and when necessary the acetic acid formed.

As special apparatus is required for the measurement of CO_2 it is much more convenient to employ method (1) where one constant suffices, as in estimating the amount of any pure substance.

In calculating the dichromate value of any aliphatic substance the rule is that the substance is completely oxidised to CO_2 except the carbon atom of a methyl group and the next adjacent carbon atom which appear as acetic acid; thus



or for each cc. *N* lactic acid we require 4 cc. *N* dichromate.

Exp. 1. 25 cc. of a lactic acid solution = 27.4 *N*/10 were mixed with 11 cc. *N* potassium dichromate, and 25 cc. concentrated sulphuric acid added rapidly. The excess dichromate was titrated with ferrous sulphate solution. Found:

27.4 cc. *N*/10 lactic acid reduced 10.77 cc. *N* dichromate;

1 cc. *N* lactic acid reduces 3.93 „

Theory 4.00 „

The experiment was repeated using twice the concentration of dichromate. Found:

27.4 cc. *N*/10 lactic acid reduced 11.27 cc. *N* dichromate;

or 1 cc. *N* lactic acid reduces 4.11 „

Theory 4.00 „

Thus even with a wide range of oxidising agent (doubling the dichromate) the reaction follows the above equation. The acetic acid produced is clearly not oxidised further, in agreement with what the writer previously found [1914].

On the other hand, substances devoid of CH_3 groups should, according to the writer's theory, yield no acetic acid but only CO_2 and H_2O . The following were tried: malonic, tartaric and citric acids, ethylene glycol; with the following results:

Exp. 2. The weight of substance mentioned in each case was dissolved in 10 cc. of water and 22 cc. *N* dichromate added. To this mixture 25 cc. concentrated sulphuric acid were added. The excess of dichromate was titrated by ferrous sulphate solution.

Results:

Ethylene glycol 0.1274 g. reduced 19.94 cc. *N* dichromate

Theory 20.55 „

Citric acid 0.0931 g. reduced 7.87 „

Theory 7.98 „

Malonic acid 0.1932 g. reduced 14.86 „

Theory 14.93 „

Tartaric acid 0.1035 g. reduced 3.71 „

Theory 3.76 „

The results are, within experimental error, in support of the theory.

The only exception to the rule seems to be succinic acid. This peculiar behaviour of succinic acid in resisting oxidation has previously been described. Under the conditions detailed above, succinic acid was not appreciably oxidised.

Exp. 3. 20 cc. *N*/20 succinic acid were mixed with 22 cc. normal potassium dichromate and to this 25 cc. concentrated sulphuric acid added. The excess of dichromate required for reduction 124.3 cc. ferrous sulphate solution; control dichromate and sulphuric without succinic acid required 126 cc.

2 cc. ferrous sulphate corresponds to 0.4 cc. dichromate and this represents an oxidation of at most 1.2 cc. of the succinic acid solution, *i.e.* 5 %. Allowing for error, we may say that from 2 to 5 % was oxidised; but the oxidation is often less than this.

By choosing the right concentration of reagents therefore it is possible to avoid the oxidation of any appreciable amount of succinic acid present. The concentration recommended is as follows:

- (a) Water containing the unknown, 10 cc.
- (b) *N* potassium dichromate 10
- (c) Sulphuric acid, conc. 20

Add (c) rapidly to the mixture of (a) and (b).

It has been shown that with the exception of succinic acid, aliphatic substances follow the rule described by the writer. We may therefore calculate directly what the oxygen equivalent of any substance will be, and it is a simple matter to construct tables for mixtures of any two substances. The following table shows the application of this principle.

Table I. *Mixture of two alcohols.*

1 cc. <i>N</i> dichromate oxidises	1/6	mol. (in mg.)	methyl alcohol or	0.00533 g.
"	"	1/4	" ethyl	" 0.0155
"	"	1/10	" propyl	" 0.0060
"	"	1/16	" butyl	" 0.004623

To draw up the table we calculate how much dichromate corresponds to one decigram of each of the two substances. The difference between these two values we may divide up into a hundred parts; we can then read off at a glance the percentage of both constituents corresponding to the dichromate titration value obtained.

Dichromate values of alcohols.

0.1 g. methyl alcohol reduces	18.740 cc. <i>N</i> dichromate
0.1 g. ethyl	" " 8.695 "
0.1 g. propyl	" " 16.670 "
0.1 g. butyl	" " 21.620 "

The marked difference between the oxygen values of methyl, ethyl and other alcohols makes the method of useful application in the case of a mixture of two alcohols of which ethyl alcohol is one.

Polyhydric alcohols also follow the same rule:

0.1 g. ethylene glycol reduces 16.120 cc. *N* dichromate

0.1 g. glycerol ,, 15.220 ,,

0.1 g. mannitol ,, 14.285 ,,

All these figures have been verified by experiment except butyl alcohol. By a simple calculation from the weight taken and the dichromate value, the proportions in the case of a mixture of two alcohols such as methyl and ethyl alcohol, or ethyl and propyl alcohol can be ascertained.

Table II. *Mixtures of any two acids.*

The acid mixture should be approximately decinormal. The mixed acids are first titrated or the titre estimated by neutralising with lime and estimating the calcium. The oxygen value of 1 cc. of a normal solution must be determined and the oxygen values of the constituents calculated from the rule given. The following values may be given as examples:

1 cc. <i>N</i> formic	acid reduces	2.00 cc. <i>N</i> dichromate
,, oxalic	,,	1.00 ,,
,, acetic	,,	0.00 ,,
,, malonic	,,	4.00 ,,
,, lactic	,,	4.00 ,,
,, succinic	,,	0.00 ,,
,, propionic	,,	6.00 ,,
,, butyric	,,	12.00 ,,
,, valerianic	,,	18.00 ,,

(or an increment of 6 cc. *N* dichromate for each CH_2 group).

Amino acids follow the same rule:

1 cc. <i>N</i> glycine	reduces	6.00 cc. <i>N</i> dichromate
,, alanine	,,	4.00 ,,

By a simple calculation the proportions of two acids in a mixture can be ascertained, provided there is a difference in their oxygen value.

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XCV. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART III. B-VITAMIN REQUIREMENTS. COMPARISON OF YEAST EXTRACTS AND DRIED YEAST.

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IN our previous experiments [1922] it was shown that chickens could be reared from the day of hatching to maturity, under laboratory conditions, if the diet contained sufficient quantities of the three vitamins; if in addition, the protein in the diet were of "good" quality, the birds reached maturity at a rapid rate. The same diet has been used by Miss Malcolm and Mrs Pember on their poultry farm in Sussex and again the rapid growth to maturity with extraordinary health and vigour was observed.

In order to make the results of greater practical value and also to ascertain the real B-vitamin requirements, the ingredients of the diet needed simplification. As a substitute for caseinogen and secwa, dried skim milk is very suitable, since it contains the same substances, caseinogen, lactalbumin and lactose and probably also the same amount of B-vitamin. The replacement of oatmeal by other cereals presents a more difficult problem. Cereal grains contain B-vitamin in different and unknown quantities, so that the amount of marmite needed to balance the carbohydrate will vary for each one. It was originally supposed that the B-vitamin in oatmeal would be destroyed in the process of kilning used in its preparation, but this required verification; further all samples of oatmeal may not be prepared in the same way. Polished white rice forms the most convenient basal cereal, as it is free from B-vitamin. The amount of B-vitamin necessary to balance this food is likely to suffice for any other cereal. Chick and Hume [1917] have already found that 1 g. of yeast extract is a sufficient daily quantity to prevent polyneuritis in pigeons on a diet of white rice, but it does not follow that this quantity will be adequate for birds of other species. As will be shown later, the requirements of chickens for B-vitamin are greater than those of pigeons.

The determination of the total amount of B-vitamin, which is necessary, reckoned in terms of yeast extract, can only be made if all the constituents of the diet are free from this vitamin. Dried skim milk is almost certain to contain B-vitamin and must be substituted by a protein without it. Such a protein is egg-white. Very little B-vitamin is present in white fish as found by Cooper [1914]. White fish meal in small quantities could therefore be used instead of egg-white.

Marmite as the source of B-vitamin is too expensive for feeding animals. A cheaper form of yeast extract, cerema, has been introduced and dried yeast is another commercial article. The relative values of these varieties of yeast need determination. Marmite has been so frequently used in scientific investigations that it may be taken as the standard for comparison. These experiments have been designed to ascertain:

- (1) the relative values of marmite, cerema, and dried yeast as a source of B-vitamin;
- (2) the amount of marmite required to balance a diet of white rice and dried skim milk;
- (3) the amount of marmite needed to balance a diet of white rice and white fish meal;
- (4) by calculation from these data, the amounts of B-vitamin, in terms of marmite, in the foodstuffs used.

Complications could arise from the different amounts of salts, fat and A-vitamin in the diets. These foods contain very little fat, so that a constant supply of fat and A-vitamin could be introduced as cod-liver oil. The salt content of the diets are almost equal. No antiscorbutic was added because there are many observations that birds can exist for a considerable time without C-vitamin. The birds of these experiments have since been kept for over a year without C-vitamin, proving it is not needed (see Part IV, p. 787).

EXPERIMENTAL.

Housing. The housing provided for the chickens has consisted of ordinary commercial appliances. Two houses of each size were procured and placed side by side so that any comparisons, which were required, could be made under the same conditions. The houses were:

- (1) three compartment foster mother, or brooder, measuring $9' \times 2' 6'' \times 1' 6''$, one of the compartments being provided with an extra metal cover and heated by a paraffin lamp;
- (2) house measuring $7' \times 5' \times 5' 6''$ with run $4' \times 5' \times 3'$;
- (3) house measuring $6' \times 4' \times 4'$ with run $4' \times 4' \times 2' 6''$ and with three outside nest boxes fitted with trap nests;
- (4) house measuring $4' \times 3' \times 4'$ with temporary run $4' \times 4' \times 2' 6''$;
- (5) house measuring $6' \times 4' \times 4'$ with temporary run $5' \times 4' \times 2' 6''$;
- (6) fireless brooder measuring $1' 4'' \times 1' 4'' \times 1' 3''$. This was placed in a larger house (2) divided by wooden partitions into two equal parts.

The houses were provided with wooden floors and peat moss litter was used as covering.

No room was available for these houses inside the laboratory. They were placed upon the flat roof of the school building. No food other than that supplied could reach the birds, but in the autumn falling leaves from adjacent trees fell upon the roof and occasionally a few were blown into the runs. In our previous experiments it was found that housing in a room or upon a roof made no essential difference to the experimental results.

The houses provided for pairs of pigeons were double wire show cages for poultry, measuring $4' \times 2' \times 2' 3''$ protected at the sides by a metal piece and covered except at the front by wood. Each cage was fitted with a central perch.

Birds. The chicks, generally started as day-old, have been most kindly placed at our disposal by Miss Malcolm and Mrs Pember from their stock at Wellingham poultry farm, Lewes, and have consisted of Light Sussex except in one experiment in which a cross Wyandotte and Light Sussex was used.

Two pairs of pigeons were procured from a dealer.

Diet. The different diets and the results of the feeding are given under the several group headings numbered in continuation of our previous work. In general, the foodstuffs were mixed together as described in our earlier papers.

Comparison of the yeast extracts, marmite and cerema, on pigeons.

In testing foodstuffs for their B-vitamin value pigeons have been the experimental animal. Chick and Hume [1917] found that 1 g. of yeast extract per day was sufficient to prevent polyneuritis on a daily diet of 40 g. of white rice. In our former experiments with chickens 0.5 g. of marmite for 30 g. of oatmeal and milk and 0.25 g. for oatmeal with caseinogen and secwa in the diet was sufficient to prevent leg weakness. It appeared in the case of growing chickens that the carbohydrate of the food needed balancing by marmite. The balancing of the carbohydrate by B-vitamin has been considered necessary by former observers, but it is difficult to prove definitely.

As a preliminary to the comparison of marmite and cerema as a source of B-vitamin for growing chickens, an experiment was started on Feb. 7th, 1922, on two pairs of adult homer pigeons. One pair, red in colour, was fed upon white rice to which were added 0.75 g. of marmite for every 90 g. of rice and 0.75 cc. of cod-liver oil. The other pair, blue in colour, was given the same diet, but 0.75 g. of cerema instead of marmite. Neither pair of birds appeared to like the diet, so that after 14 days the cod-liver oil was omitted. On Feb. 24th, *i.e.* in the third week, both hens showed signs of leg weakness and a high stepping gait and were unable to get upon the perch; both cocks were also unwell. Their feathers were ruffled. Each bird was therefore dosed with 0.25 g. of marmite, or of cerema, dissolved in about 5 cc. of water on two consecutive days. This caused improvement in all the birds. The quantity of marmite, or of cerema, in the food was therefore not sufficient and was raised

on Feb. 27th to 1.5 g. per 90 g. of rice. This additional quantity did not, however, prevent the birds from getting ill again and they were all dosed with 0.5 g. of cerema, or of marmite, for three consecutive days. Their condition improved and their appetite increased. The marmite and cerema were both raised to 3 g. per 90 g. of rice on Mar. 7th.

A diet consisting only of polished rice and marmite, or cerema, cannot be considered adequate without additional protein, salts and A-vitamin. Fish meal (sample 1 see p. 785) was added on Mar. 11th.

On this diet, consisting of 90 g. white rice, 15 g. fish meal and 3 g. of marmite, the red pair of birds improved, gained in weight and the hen laid eggs on Apr. 5th. The blue pair of birds with 3 g. of cerema in the place of marmite did not show a similar improvement: their appetite fell off and they looked unwell with ruffled feathers. They were given a dose of 1 g. cerema on Mar. 27th and at the same time the amount of cerema in the food was raised to 3.75 g. per 90 g. of rice. This amount again did not suffice. The hen bird on Apr. 4th showed the characteristic symptoms of polyneuritis. She recovered after three doses of 1 g. cerema and was able to walk about the next day. On Apr. 12th the cerema was raised to 6 g., and on Apr. 23rd to 7.5 g. per 90 g. rice. The original supply of cerema was now exhausted. The new supply was quite different in appearance and it was learned that the original was from some old stock. The experiment was therefore stopped and both pairs of birds placed on oatmeal and fish meal to ascertain if C-vitamin was needed in their food (see Part IV, p. 787).

Thus, as far as marmite is concerned, 3 g. per 90 g. rice is sufficient to keep pigeons in health. The food consumption was on the average 45 g. rice and 7.5 g. fish meal per day. This is rather more than that given by Chick and Hume.

It may be thought that the salt addition with the fish meal was the reason for the maintenance of the red pair of pigeons, but this does not explain the failure of the blue pair on exactly the same diet.

With insufficient marmite or cerema not only did the appetite fall off, but also the weights. There was an increase of appetite after dosing and increase in weight. A steady increase in weight was shown by the red pair as soon as the balance of rice and marmite was reached. The weights are given in the following table:

	Red pair		Blue pair	
	Cock	Hen	Cock	Hen
	g.	g.	g.	g.
Feb. 9	445	439	434	327
" 15	435	430	429	318
" 22	409	391	392	286
Mar. 1	366	392	363	273
" 8	354	366	347	281
" 16	368	364	353	260
" 22	414	414	365	281
" 29	442	467	368	311
Apr. 7	438	450	407	321
" 19	447	472	398	284

GROUP IV. *Value of cerema and rice diet.*

Groups I to III of chickens had been kept on oatmeal and milk, or oatmeal and caseinogen and secwa with 0.75 g. of marmite per 90 g. of oatmeal and the results reported in our former papers [1922].

This experiment with Group IV was made to test the value of cerema as a source of B-vitamin for chickens. At the same time white rice was substituted for oatmeal and dried skim milk for caseinogen and secwa.

Twelve day-old Light Sussex chicks were used and the experiment was started on Feb. 22nd, 1922.

The diet was composed of equal parts of white rice and of dried skim milk. A-vitamin was added as cod-liver oil, at first 0.25 cc. but after the tenth day 0.5 cc. per bird per day. B-vitamin was included as cerema in the proportion of 0.75 g. per 90 g. of rice, the same quantity as the marmite used for oatmeal and milk. C-vitamin was not added as it was desired to see if chickens could be reared without it (see Part IV). During the second and third weeks the appetite of the birds was not good and it was not necessary to increase the daily food supply as often as expected. During the fourth week some slight signs of leg weakness were evident. On Mar. 30th at the beginning of the fifth week most of the birds showed very distinct signs of leg weakness. The amount of cerema in the food was then doubled. In addition, each bird was given a dose of 1 g. of cerema dissolved in about 5 cc. of water. This dosing was repeated on Apr. 4th and the amount of cerema in the food raised to 3 g. per 90 g. of rice. After a third dose the birds got slightly better, but still remained weak. Cerema was raised on Apr. 6th to 4.5 g. and on Apr. 11th to 6 g. per 90 g. of rice. The improvement was not maintained in spite of occasional doses of 1 g. of cerema. Ten birds died between Apr. 10th and May 19th. The remaining two recovered after raising the cerema to 7.5 g. per 90 g. of rice and were kept to maturity.

No definite result is given by the experiment, but it indicates that either more B-vitamin is required to balance rice than oatmeal, if cerema and marmite are equivalent, or cerema has less B-vitamin than marmite.

The record of the food consumption and weights of the birds is of no value.

GROUP V. *Value of cerema and oatmeal diet.*

The failure to rear the chickens of Group IV on equal parts of white rice and dried skim milk with the addition of 0.75 g. of cerema per 90 g. of rice led to a return to oatmeal as carbohydrate. Equal parts of oatmeal and dried skim milk give a food mixture of the composition:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
8.1 %	4.7 %	0.5 %	21.9 %	4.3 %	60.6 %

with a protein to fat and carbohydrate ratio of 1 : 3.

This mixture corresponded fairly closely with the original mixture of

5/7 oatmeal, 1/7 caseinogen and 1/7 secwa used in the latter part of the experiment with Group III, namely

Water	Ash	Fibre	Protein	Fat	Carbohydrate
7.0 %	3.7 %	0.7 %	22.4 %	6.1 %	60.2 %

and protein to fat and carbohydrate ratio of 1 : 3.

Cod-liver oil was added to supply A-vitamin, at first at the rate of 0.5 cc., later 1.5 cc. per bird per day. Cerema was added to supply B-vitamin in the proportion of 0.75 g. per 90 g. of oatmeal. Orange juice was included for the first 37 days at the rate of 1 cc. per bird per day; it was afterwards omitted. Ten day-old Light Sussex chicks were used and the experiment started on May 4th, and concluded on Aug. 2nd, 1922.

Except for the accidental loss of five birds during the second week, owing to the paraffin lamp of the brooder smoking, the birds were raised to maturity without failure. The five dead birds were examined and no lesion could be discovered other than the lungs being filled with soot.

The food consumption and growth, owing to the accident, cannot be considered normal during the second and third weeks as all the birds were affected by the soot. The record is as follows:

		Oat- meal	Dried skim milk	Cod- liver oil	Cerema	Orange juice	Average weekly weights:		
		g.	g.	cc.	g.	cc.	g.	g.	g.
May	5	30	30	2.5	0.25	10	May	4	39.4
"	6-29	60	60	5.0	0.5	10	"	10	59.2
"	30-June 1	90	90	7.5	0.75	5	"	17	91
June	2-9	120	120	7.5	1.0	5	"	24	155
"	10-14	120	120	7.5	1.0	0	"	31	228
"	15	150	150	7.5	1.25	0	June	7	295
"	16-26	180	180	7.5	1.5	0	"	14	421
"	27-July 10	270	270	7.5	2.25	0	"	21	560
July	11-Aug. 3	360	360	7.5	3.0	0	"	28	736
							July	1	806
							"	12	1099
							"	19	1282
							"	27	1471
							Aug.	2	1648

No comparison of the rate of growth can be made with the birds of Group III which were white Leghorns.

On a carbohydrate basis of oatmeal 0.75 g. of cerema is sufficient per 90 g. This is the same as the quantity of marmite used in Group III. Cerema and marmite thus appear of equal value as a source of B-vitamin, if the amounts of B-vitamin in dried skim milk and in caseinogen and secwa are the same.

GROUP VI. *Comparison of marmite and cerema on chickens.*

The experiment with Group IV had shown that the addition of 0.75 g. of cerema per 90 g. of rice was not enough to maintain the birds. The experiment of Group V with 0.75 g. cerema per 90 g. of oatmeal indicated that this extract had an equal value to marmite. A thorough comparative experiment was now made on two lots of birds simultaneously, the one lot with cerema, as source of B-vitamin, and the other lot with marmite. The experiment with pigeons had shown that 3 g. of marmite per 90 g. of white rice was sufficient. This quantity of each extract was therefore included in the diet. Cod-liver oil to supply A-vitamin was added in the proportion of 0.5 cc. per bird per day. C-vitamin was omitted.

The diet was composed of equal parts of white rice and dried skim milk and had the following composition:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
10.0 %	4.1 %	0.2 %	19.1 %	0.3 %	66.3 %

with a protein to fat and carbohydrate ratio of 1 : 3.5.

The experiment was started on June 26th, and concluded on Dec. 19th, 1922, at which date it was decided to put the hens and one cock into a single group on the diet with marmite for the purpose of procuring a second generation on a C-vitamin free food mixture (see Part IV, p. 787).

The constituents of the diet remained the same throughout; the only alteration was a reduction of the dried skim milk to 1/3 of the total on Aug. 21st (end of 8th week), mainly for the purpose of lessening the cost of feeding. The composition of the food mixture then became:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
10.5 %	2.9 %	0.2 %	14.9 %	0.4 %	71.2 %

with a protein to fat and carbohydrate ratio of 1 : 4.8.

The group consisted of two lots of ten day-old chicks Wyandotte crossed with Light Sussex. Of these twenty birds, only two did not reach maturity. Both belonged to the cerema lot. One died on the 9th day of the experiment; no reason could be found on post mortem examination. The other was found paralysed in one leg at the end of the 9th week; post mortem examination indicated that this was due to pressure by a fibrous ovary on a nerve. On Sept. 28th, at the end of the 13th week, both groups showed signs of slight leg weakness. The birds were always wanting to rest and some of the cocks had a slight high stepping gait. Both the cerema and marmite were then raised, the marmite to 3.75 g. and the cerema to 4.5 g. per 90 g. of rice. The reason for the higher increase of cerema was because the birds of this lot seemed slightly worse. The symptoms disappeared in both lots in about 14 days.

Cocks of each lot started to crow during the 16th week.

On Nov. 11th (20th week) the birds of the cerema lot again showed slight signs of leg weakness. The cerema was then increased to 5.0 g. per 90 g. of rice. This figure was chosen so as to correspond with the nitrogen content of the preparations: marmite 5.7 %, cerema 4.3 %. After raising the cerema the symptoms disappeared.

On Nov. 15th (21st week) it became essential to separate the hens and cocks; the cerema lot became one cock and three hens, the marmite lot one cock and four hens. Excess cocks were kept in their separate lots. The hens of the cerema lot started laying on Dec. 10th (24th week), those of the marmite lot on Dec. 16th.

The total number of hens was too small to continue as two breeding pens. One cock and the seven hens were made into a single group and the experiment as regards comparison of cerema and marmite was terminated. The birds

were kept longer for more evidence about C-vitamin and its non-requirement by chickens (see Part IV, p. 787).

The two yeast extracts appear to be of nearly equal value; the two losses in the cerema lot cannot be fairly attributed to the presence of less B-vitamin. The early loss may have been due to a weaker chick at the start, the later one to some fault in sexual development. 5.0 g. of cerema is the equivalent of 3.75 g. of marmite per 90 g. of white rice with dried skim milk in the food. Throughout, the daily food consumption and the average weekly weights were very similar, as seen from the tables:

Daily food consumption.

	Rice g.	Dried milk g.	Cerema g.	Cod- liver oil cc.		Rice g.	Dried milk g.	Mar- mite g.	Cod- liver oil cc.
June 26	30	30	1.0	5	June 26	30	30	1.0	5
" 27-July 7	60	60	2.0	5	" 27-July 7	60	60	2.0	5
July 8-12	120	120	4.0	5	July 8-12	120	120	4.0	5
" 13-20	150	150	5.0	5	" 13-19	150	150	5.0	5
" 21-23	180	180	6.0	5	" 20	180	180	6.0	5
" 24	240	240	8.0	5	" 21-23	240	240	8.0	5
" 25-Aug. 2	270	270	9.0	5	" 24-31	270	270	9.0	5
Aug. 3-6	300	300	10.0	5	Aug. 1-2	300	300	10.0	5
" 7-8	330	330	11.0	5	" 3-8	330	330	11.0	5
" 9-11	360	360	12.0	5	" 9-11	360	360	12.0	5
" 12-13	390	390	13.0	5	" 12-13	390	390	13.0	5
" 14-20	420	420	14.0	5	" 14-16	420	420	14.0	5
" 21-Sept. 6	570	285	19.0	5	" 17-20	450	450	15.0	5
Sept. 7-25	780	390	26.0	5	" 21-Sept. 6	600	300	20.0	5
" 26-27	840	420	28.0	5	Sept. 7-25	810	405	27.0	5
" 28-30	840	420	35.0	5	" 26-27	870	435	29.0	5
Oct. 1-24	840	420	42.0	5	" 28-Oct. 24	870	435	36.25	5
" 25-31	900	450	45.0	5	Oct. 25-31	930	465	38.75	5
Nov. 1-10	960	480	48.0	5	Nov. 1-8	990	495	41.25	5
" 11-Dec. 17	960	480	52.5	5	" 9-Dec. 17	1050	525	43.75	5

Average weekly weights.

	Cerema lot g.	Marmite lot g.		Cerema lot g.	Marmite lot g.
June 26	40	40	Aug. 7	549	498
July 3	76	75	" 10	628	579
" 10	132	131	" 21	845	797
" 17	215	200	" 28	988	972
" 24	282	283	Sept. 4	1140	1123
" 31	431	390	" 11	1279	1307
			" 25	1595	1653

	Cerema lot		Marmite lot	
	Cocks	Hens	Cocks	Hens
	g.	g.	g.	g.
Oct. 2	1927	1479	2062	1426
" 9	2143	1594	2302	1572
" 16	2351	1688	2486	1659
" 23	2424	1713	2615	1700
" 31	2701	1815	2876	1847
Nov. 8	2732	1872	2983	1931
" 15	2862	1952	3032	1986
" 22	2930	1985	3055	1993
Dec. 4	3070	2082	3198	2030
" 11	3108	2225	3240	2090
" 18	3192	2346	3288	2141

GROUP VII. *Comparison of cerema and dried yeast.*

Two lots of ten day-old Light Sussex chicks were used for this comparison, which was started on Dec. 22nd, 1922. The food for each lot consisted of two-thirds white rice and one-third dried skim milk. Cod-liver oil was added to each daily food supply at the same rate as before, 5 cc. per day, or 0.5 cc. per bird. C-vitamin was omitted. One lot received cerema in the proportion of 6 g. per 90 g. of rice. This quantity was chosen so as definitely to avoid any signs of leg weakness and was rather greater than the amount of 5 g. previously found to be sufficient. The other lot received 6 g. of dried yeast per 90 g. of rice. The two diets are not exactly equivalent on account of the higher nitrogen or protein content of dried yeast, 7.6 % against 4.3 % of cerema. This was unavoidable, as no similar protein could be added to compensate.

On Jan. 16th, 1923, the birds of the dried yeast lot showed distinct signs of leg weakness and drooping wings. The dried yeast was therefore raised to 12 g. per 90 g. of rice. On Apr. 6th, in the 15th week of the experiment, some signs of leg weakness became noticeable in the cerema lot; the amount of cerema was raised to 6.75 g. per 90 g. of rice. No other alteration was made and the experiment was terminated on Apr. 14th. Cocks of each group began to crow about Mar. 25th. The birds were mature and the hens needed separation from the cocks. The hens and a cock were continued as a single group for ascertaining egg production and hatchability on the C-vitamin-free diet (see Part IV). The first egg was laid on Apr. 29th in the 20th week.

Daily food consumption.

	Rice	Dried skim milk	Cerema	Cod-liver oil		Rice	Dried skim milk	Dried yeast	Cod-liver oil
	g.	g.	g.	cc.		g.	g.	g.	cc.
Dec. 22-27	60	30	4	5	Dec. 22-25	60	30	4	5
" 28	90	45	6	5	" 26-28	90	45	6	5
" 29-Jan. 7	120	60	8	5	" 29-Jan. 2	120	60	8	5
Jan. 8-9	150	75	10	5	Jan. 3-7	150	75	10	5
" 10-11	180	90	12	5	" 8-9	180	90	12	5
" 12-14	210	105	14	5	" 10-11	210	105	14	5
" 15-19	240	120	16	5	" 12-14	240	120	16	5
" 20-24	270	135	18	5	" 15-17	270	135	36	5
" 25-29	300	150	20	5	" 18-19	300	150	40	5
" 30-31	330	165	22	5	" 20-22	330	165	44	5
Feb. 1-7	360	180	24	5	" 23-25	360	180	48	5
" 8-9	390	195	26	5	" 26-29	390	195	52	5
" 10-13	420	210	28	5	" 30-31	420	210	56	5
" 14	450	225	30	5	Feb. 1-19	450	225	60	5
" 15-19	480	240	32	5	" 20	480	240	64	5
" 20	510	255	34	5	" 21-23	540	270	72	5
" 21-23	540	270	36	5	" 24-26	600	300	80	5
" 24-26	600	300	40	5	" 27-Mar. 6	660	330	88	5
" 27-Mar. 6	660	330	40	5	Mar. 7-30	720	360	96	5
Mar. 7-Apr. 5	720	360	48	5	" 31-Apr. 13	780	390	104	5
Apr. 6-13	720	360	54	5					

Altogether three birds out of 20 were lost. One bird of the cerema lot died on the second day of the experiment. Another bird of this lot dislocated

its leg in the second week and was chloroformed. The third bird belonged to the dried yeast group and was found dead on Feb. 6th in the 7th week; it had suffered more severely than the other birds from insufficiency of dried yeast before the quantity was raised and owing to its weakness may have been crushed by the birds huddling together at night. Excluding the accidental loss, 90% of each lot was raised to maturity. Just before the conclusion of the experiment, on Apr. 7th, a cock of the cerema lot was found with a damaged leg, evidently from fighting and was chloroformed.

The close parallelism between the two lots is shown by the daily food consumption (p. 780) and the weekly average weights of the birds which were:

	Cerema lot	Dried yeast lot		Cerema lot	Dried yeast lot
	g.	g.		g.	g.
Dec. 22	37.6	36.7	Feb. 23	921	992
" 29	76.6	74.8	Mar. 2	1072	1089
Jan. 5	132.4	137.6	" 9	1262	1334
" 12	220	234	" 16	1418	1508
" 19	284	309	" 23	1596	1598
" 26	386	400	" 30	1801	1840
Feb. 2	514	502	Apr. 6	1920	2052
" 9	604	665	" 13	2034	2144
" 16	748	810			

The higher consumption in the dried yeast lot was due to its having one or two more birds at certain periods, as the result of losses in the cerema lot. The food consumption per bird is approximately the same in both lots.

The advantage, if any, is on the side of the dried yeast lot. The two lots contained the same number of cocks and hens.

It is interesting to note that these weights run very close to the weights of the birds in Group VI. Group VI was started in June, Group VII in December. There is no appreciable difference in the rate of growth at different seasons of the year, as is commonly believed by poultry keepers. The result shows that a proportion of 12 g. of dried yeast is equal to 6, or more probably, 6.75 g. of cerema per 90 g. rice on a diet of two-thirds white rice and one-third dried skim milk.

GROUPS VIII and IX. *Amount of marmite required for a diet containing no B-vitamin.*

In order to determine the full amount of marmite which must be included in a diet not containing B-vitamin in any of its ingredients, it was necessary to replace the dried skim milk in the diet of the previous groups by some protein with little or no B-vitamin. Cooper [1914] has shown that more than 10 g. per day of white fish was required to prevent polyneuritis in pigeons. The total amount necessary was not determined, but this result suggests that white fish flesh has very little B-vitamin. Dried commercial white fish meal thus seemed a suitable protein for these experiments, if used in small amounts. 5 g. of fish meal with 90 g. of rice would not be likely to introduce an appreciable amount of B-vitamin.

Fish meal contains a large proportion of mineral matter, so that a further ash supplement is unnecessary. Some samples of fish meal have a low fat content (see p. 785 for analyses) and the amount of A-vitamin in this fat is probably small. Fish meals, dark in colour, are evidently made at a high temperature and some, if not all, of the A-vitamin would be destroyed. A known amount of fat supplying A-vitamin in the form of cod-liver oil was therefore added.

The quantity of marmite to be included as source of B-vitamin required consideration. Group VII showed that 6 g. of cerema or 12 g. of dried yeast was adequate for 90 g. of rice and 30 g. of dried skim milk. This amount was likely to be too small on the fish meal diet, since the dried skim milk probably carried enough B-vitamin to balance its carbohydrate, fat and protein. The dried skim milk supplied 28 parts of combustible food material and the rice supplied 58 parts. Half as much cerema again would be required to balance the 28 parts derived from the dried skim milk, now replaced by fish meal and a proportion of rice. Consequently for the fish meal diet containing 90 g. of rice and 5 g. of fish meal $6 + 3 = 9$ g. of cerema would be needed. Cerema and marmite are nearly equal, so 9 g. of marmite was added to balance these amounts and a small allowance of cod-liver oil, 0.5 cc. per 90 g. of rice.

The diet composed of 90 g. of rice, 5 g. of fish meal (sample 2), 0.5 cc. of cod-liver oil and 9 g. of marmite had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
12.7 %	1.7 %	2.7 %	0.3 %	11.3 %	1.0 %	70.6 %

with a protein to fat and carbohydrate ratio of 1 : 6.3. Marmite is present in the proportion of 8.6 %.

In order to ascertain if the amount of marmite was properly adjusted, the cod-liver oil was raised at intervals to 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 cc. At the same time the effect of the extra cod-liver oil and its relation to the amount of B-vitamin would be shown. Our previous experiments (Part I) had indicated that extra cod-liver oil needed balancing with B-vitamin. This question is being further studied in another group of birds.

GROUP VIII consisted of 11 Light Sussex birds, 7 weeks old, previously kept on a rice and dried skim milk diet as used for Group VII. The experiment was started on Mar. 9th and concluded on June 21st, 1923 (14 weeks). The birds did not grow rapidly and there was considerable difference in the weights of the birds of the same age and sex, particularly noticeable after the oil was raised to 2.5 and 4 cc. Some of the hens became adult long before others. One of them had a sort of spinal curvature. The birds were not active and always wanted to rest. Signs of leg weakness with high stepping gait and drooping wings were observed. Their feathering was slow and their combs were pale. The cocks were never heard to crow. All the birds were raised to maturity. The general appearance of the birds was the best indication that the diet contained just the sufficient amount of B-vitamin to maintain them and take them to maturity, but not enough to keep them in

good health. The conditions of the experiment were repeated in the next group.

GROUP IX consisted of 10 Light Sussex day-old chicks, which were fed on the same diet as Group VIII. The experiment was started on Apr. 7th and concluded on Aug. 24th, 1923 (20 weeks).

Between May 4th and June 19th, the cod-liver oil was gradually raised from 0.5 cc. to 4 cc. (see table of weights). With the increase of the cod-liver oil the birds had the appearances of those of Group VIII, pale combs, slight leg weakness, high stepping gait, poor feathering, which pointed to an insufficiency of B-vitamin in the diet. The marmite was therefore raised on June 30th (13th week) to 9.75 g. per 90 g. rice. Some improvement followed. It was raised again on Aug. 3rd (17th week) to 10.5 g. per 90 g. of rice. The general improvement was more noticeable.

The food intake was not measured daily as in other groups. The food mixture was made up in sufficient quantity to last for two or three days. The birds were allowed as much as they would eat; food was constantly before them. The actual consumption has no particular bearing on the observations.

Three birds were lost during the experiment. One was unwell on arrival and died the first day. The second died on June 20th. This bird had the most severe symptoms of leg weakness in the group. Post mortem examination showed considerable abnormality of the gut, the walls of which were very thick. The third died on Aug. 7th. This bird had stoppage and distention of the gut at the junction of the caeca. The connective tissue holding the intestines was very tight and fibrous.

One of the birds raised to maturity showed some deformity in one wing, probably from resting on it during the time the leg weakness was observed.

It is not possible to compare the growth with that of the birds of Groups VI and VII, which had not only more protein, but also milk protein in the diet. In order to see the irregularity the weekly weight record of each bird must be recorded (see p. 784).

The weekly weights are not the best indication of the condition of the birds. The condition can really only be judged by comparison with other birds which have been reared on a diet containing all the constituents in better proportions. At 20 weeks the birds were not mature. The cocks never crowed and the hens were small for their age. It was difficult to be certain of the sex of two of the birds, Nos. 2 and 6. Only at the end did their sex become quite certain.

The results confirm those of Group VIII. On a diet of 90 g. rice, 5 g. fish meal and 0.5 cc. cod-liver oil, 9 g. of marmite is sufficient to maintain and raise them to maturity. Excess of cod-liver oil produced signs of leg weakness. These observations do not agree with those of Emmett and Peacock [1923] who attribute leg weakness and drooping wings to lack of A-vitamin. A large excess of cod-liver oil was present in the diets of Groups VIII and IX with scarcely enough marmite to balance the rice and fish meal adequately. Our

result suggests that the diet used by Emmett and Peacock had not enough B-vitamin in it. Perfect health and growth depend upon a proper amount of all the constituents in the diet. The question of the balance of carbohydrate, fat and protein with B-vitamin is now under investigation.

Average weekly weights, and time of increase of cod-liver oil and marmite.

	Apr. 7	Apr. 13	Apr. 20	Apr. 27	May 4	May 11	May 18	Sex
Cod-liver oil	0.5 cc.	0.5 cc.	0.5 cc.	0.5 cc.	1 cc.	1 cc.	2 cc.	
Marmite	9.0 g.	9.0 g.	9.0 g.	9.0 g.	9.0 g.	9.0 g.	9.0 g.	
No. 1	41.5 g.	59.5 g.	95.0 g.	150 g.	205.0 g.	303.0 g.	385 g.	Cock
" 2	41.0	66.0	82.0	123	182.0	230.0	317	Cock
" 3	41.0	62.5	83.0	125	152.0	221.0	293	Cock
" 4	42.5	63.5	84.0	129	173.0	242.0	329	Hen
" 5	39.0	61.5	82.0	117	157.0	219.0	292	Hen
" 6	38.5	59.5	84.0	115	149.0	202.0	250	Cock
" 7	40.0	53.5	64.0	88	111.0	159.0	203	Hen
" 8	40.5	63.5	81.0	109	139.0	190.0	238	
" 9	38.5	49.5	58.0	80	97.0	134.0	184	
" 10	41.0	Died						
Average	40.3	59.7	79.9	115	151.5	211.0	277	
Increase	—	19.4	20.2	35	36.5	59.5	66	

	May 25	June 1	June 8	June 15	June 22	June 29	July 6	Sex
Cod-liver oil	2.5 cc.	2.5 cc.	2.7 cc.	3.5 cc.	4.0 cc.	4.0 cc.	4.0 cc.	
Marmite	9.0 g.	9.0 g.	9.0 g.	9.0 g.	9.0 g.	9.75 g.	9.75 g.	
No. 1	519 g.	580 g.	800 g.	905 g.	1140 g.	1325 g.	1526 g.	Cock
" 2	398	520	682	802	935	1100	1241	Cock
" 3	393	555	675	823	975	1165	1314	Cock
" 4	429	525	635	777	957	1105	1257	Hen
" 5	365	495	605	690	840	915	1055	Hen
" 6	334	425	505	603	680	765	863	Cock
" 7	253	330	352	422	488	555	612	Hen
" 8	312	417	498	568	665	775	854	
" 9	234	300	402	517	Died			
Average	360	494	573	678	835	963	1080	
Increase	83	134	79	105	157	128	117	

	July 13	July 20	July 27	Aug. 3	Aug. 10	Aug. 17	Aug. 24	Sex
Cod-liver oil	4.0 cc.	4.0 cc.	4.0 cc.	4.0 cc.	4.0 cc.	4.0 cc.	4.0 cc.	
Marmite	9.75 g.	9.75 g.	9.75 g.	10.5 g.	10.5 g.	10.5 g.	10.5 g.	
No. 1	1640 g.	1900 g.	1940 g.	2170 g.	2250 g.	2450 g.	—	Cock
" 2	1312	1480	1605	1730	1940	2100	—	Cock
" 3	1410	1515	1600	1800	1950	2025	—	Cock
" 4	1207	1400	1560	1725	1890	1960	—	Hen
" 5	1127	1198	1280	1420	1570	1675	—	Hen
" 6	920	1012	1122	1235	1325	1480	—	Cock
" 7	659	700	725	800	990	995	—	Hen
" 8	922	919	857	905	Died			
Average	1149	1265	1336	1473	1702	1812		
Increase	69	118	71	137	229	110		

The comparative B-vitamin value of foodstuffs.

The comparative value of several foodstuffs in preventing polyneuritis in pigeons has been determined by Chick and Hume [1917] and Cooper [1914]. Yeast extract (marmite) was found to contain more B-vitamin than any of the others except rice germ. The B-vitamin content of marmite has also been found to be very constant. The constancy has again been shown by our experiments with pigeons (p. 774). For purposes of comparison, marmite may be chosen as the most convenient standard. If the figure of 1 g. found by

the above observers be put at 100, their figures give the following comparative numbers:

Marmite	100	Ox liver	33	Ox heart muscle	20
Wheat germ	67	Barley, unhusked	27	Ox brain	17
Pressed yeast	40	Barley, husked	20	Sheep brain	8
Lentils	33	Peas	20	Beef muscle	5
Egg yolk	33				

Groups VI and VII showed that 1.25 g. marmite were equal to 1.66 g. of cerema and 2 to 2.25 g. of cerema were equal to 4 g. of dried yeast. Hence, if marmite = 100, cerema = 75, dried yeast = 50.

Groups VIII and IX were raised to maturity on the diet of 90 g. of rice and 5 g. of fish meal with the addition of 9 g. of marmite, or 8.6 % of the total food.

Group VI, on a diet of equal parts of rice and dried skim milk, needed an addition of 3 g. of marmite per 90 g. of rice. Assuming that 90 g. of dried skim milk require 9 g. of marmite, as only 3 g. were wanted, this quantity of dried skim milk would contain the equivalent of 6 g. of marmite, or 6.7 %.

It is not possible to calculate from the diet of Group V, equal parts of oatmeal and dried skim milk, the B-vitamin value of oatmeal; but in a further experiment (Group XI, see Part V) chicks were raised to maturity on a diet of 90 g. of oatmeal and 5 g. of fish meal with an addition of 1.5 g. of marmite. The 95 g. of food would thus contain an equivalent of $9 - 1.5 = 7.5$ g. of marmite or 7.9 %.

Oatmeal, as a cereal, thus contains a considerable amount of B-vitamin, but it is not sufficient for the rearing of chicks. It does not follow that whole oats contain the same amount, nor that other cereals and cereal products have an equal amount. Germ meals may contain more. Further feeding experiments will be necessary to ascertain the amounts. In the meanwhile, in order to be on the safe side for maintaining the health of chicks, the addition of some foodstuff rich in B-vitamin is advisable. The maximum needed would be 8.6 % in terms of marmite or 17 % in terms of dried yeast. With a cereal such as oatmeal an addition of 1.5 % of marmite or 3 % of dried yeast would be adequate.

Analyses of foods.

The foods which have been used in these experiments had the following composition, from which the composition of the actual diets have been calculated:

	Water %	Salt %	Other ash %	Fibre %	Protein %	Fat %	Carbohydrate	
							By diff. %	Undet. %
Oatmeal*	7.5	—	1.9	1.0	12.0	8.4	69.4	—
Rice white*	11.4	—	0.6	0.3	6.5	0.4	80.8	—
Dried skim milk (1)	7.0	—	7.6	—	31.8	0.1	43.3†	10.2
" " (2)	8.6	—	7.6	—	31.8	0.2	43.4†	8.4
Fish meal (1)	14.7	3.1	18.6	—	60.5	3.3	—	—
" " (2)	12.1	3.2	21.3	—	59.8	1.9	—	—
Marmite	27.3	14.5	13.9	—	32.5	—	11.8	—
Cerema	28.6	6.9	10.5	—	24.4	—	29.6	—
Dried yeast	2.5	—	8.6	—	43.5	0.9	44.5	—

* From *Analyses and Energy Values of Foods* by R. H. A. Plimmer.

† Lactose by analysis.

SUMMARY.

1. Pigeons on a diet of polished rice (90 g.) and fish meal (15 g.) need an addition of 3 g. of marmite for maintenance.

2. Chicks on a diet of equal parts of white rice and dried skim milk need an addition of 3 g. of marmite per 90 g. of rice, or 3.3 %.

3. Chicks on a diet of equal parts of oatmeal and dried skim milk need an addition of 1.5 g. of marmite per 90 g. of oatmeal, or 1.6 %.

4. Chicks on a diet of 90 g. of rice and 5 g. of fish meal with 0.5 cc. of cod-liver oil need an addition of 9 g. of marmite, or 8.6 % of the food.

5. If marmite be put at 100, cerema has a value of 75, dried yeast of 50, oatmeal of 7.9, dried skim milk of 6.7.

We gratefully acknowledge supplies of marmite, cerema and dried yeast from the Marmite Food Extract Co., Ltd.

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XCVI. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART IV. C-VITAMIN REQUIREMENTS OF CHICKENS AND OTHER BIRDS.

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THE growth of the chick in the shell to the day of hatching is one of the best indications that C-vitamin is not needed by the growing bird, since there is no evidence to show that the contents of eggs have any anti-scorbutic value. Further, in those species of animals which suffer from scurvy, the young are most quickly affected. These considerations suggested to us that birds would not require C-vitamin at any time during their lives. The earlier workers upon beri-beri maintained chickens for many weeks upon a diet free from C-vitamin. More recently, Shorten and Ray [1921] kept fowls for 13 weeks on a diet of dried vegetables, proved by experiments on guinea-pigs to be free from C-vitamin, but their birds died from some unknown causes.

Sugiura and Benedict [1923] have kept pigeons on a diet without C-vitamin for 147 days. They consider also that A-vitamin is not required by pigeons (or at most only a very small amount). The birds produced eggs and raised their young on the same diet. Chickens have been kept by Mitchell, Kendall and Card [1923] for about 20 weeks on a diet not containing C-vitamin; the addition of food with C-vitamin to a control set of birds made no appreciable difference to the rate of growth. These observers, and also Emmett and Peacock [1923], find that A-vitamin is needed in considerable amount by chickens.

In view of the fact that scurvy, which is due to the absence of C-vitamin, does not appear in man for a period of four to six months, though guinea-pigs succumb in three to four weeks, a period of 20 weeks can hardly be considered sufficiently long to prove that chickens do not need C-vitamin. At any rate birds should be maintained on such a diet for twice as long as the fatal period in man, i.e. for 8 to 12 months. This long period was designed for our experiments and in that time it was expected to raise another generation. The

experiments have been extended to pigeons and other birds. Though success in raising a new generation has been very limited, which appears to be connected with some other deficiency in the diet, it may still be concluded that chickens, pigeons and other birds never seem to require the addition of C-vitamin to their food. The addition of green food to the food of birds so constantly advised by poultry keepers is not for the C-vitamin which it contains.

EXPERIMENTAL.

Chickens.

The birds used in these experiments were those of Group VI and also Group VII. The pigeons were those also described in Part III [1923]. The other birds were ducks, geese, guinea-fowl, turkeys and pheasants. With these latter birds the main experiment was in connection with their comparative B-vitamin requirements (see Part V, p. 794).

Group VI. The chickens of this group were formed into a breeding group on Dec. 19th, 1922, and kept until Aug. 10th, 1923, at which date the experiment was concluded. Throughout the whole period lasting for nearly 14 months these birds were given the same diet of white rice and dried skim milk supplemented with marmite and cod-liver oil.

Eggs were laid regularly from Dec. 1922 to Aug. 1923. The egg record is not of any value, since many eggs were laid in the house, or run, and were eaten by the birds. In order to stop the egg eating, the amount of marmite in the diet was raised on Jan. 15th to 4.5 g., on Feb. 1st to 5.25 g., on Feb. 16th to 6 g., on Mar. 13th to 6.75 g. per 90 g. of rice or 120 g. of food. This did not altogether stop the egg eating.

The hatchability of the eggs was not tested till Feb. 5th. Those laid between this date and Mar. 7th were not fertile. Most of the eggs laid between Mar. 7th and Apr. 9th were unfertile, but five had germs; these were not strong enough to hatch.

The A-vitamin content of the eggs was now investigated by Miss Coward and Mr Channon, whose results will be published shortly. The A-vitamin content was found to be below normal. In consequence of this deficiency the amount of cod-liver oil in the diet of the birds was raised from 0.5 to 2 cc. per bird per day on Apr. 18th.

Eggs tested at the end of April and beginning of May showed greater fertility, but still did not hatch. Many of them had well developed chicks. This ill success in hatching may have been due in part to the incubator, which was of the laboratory pattern not adapted for hatching eggs.

It became possible to test the hatchability of eggs laid in June under a broody hen. Out of three eggs tested one hatched out and has been reared successfully to the middle of August. The other two eggs were not fertile and were laid by a hen whose eggs had throughout been unfertile.

At the same time as these eggs were put under a broody hen, two eggs from a hen of Group VII were also tried; both were fertile and chicks hatched. A second generation has thus been procured from birds whose diet has not contained C-vitamin. As Group VII had given eggs from which birds could be hatched it became unnecessary to keep the birds of Group VI after they had been on a diet free from C-vitamin for about 14 months. The group was therefore got rid of.

During the course of the experiment one of the hens was found dead on inspection in the morning. No actual cause could be found on post mortem examination. An egg was found fully formed in the oviduct and death was probably due to its being egg bound.

Two hens died in July. One of them, an excellent egg layer, had its legs broken probably by the cock and evidently died of the shock. The other never laid an egg; like the hen which died at the end of the ninth week it was found to have a fibrous ovary.

Two other hens were bad egg layers. One laid a few eggs in January. The other, as far as we were able to notice, never laid an egg, though she may have laid in the run and eaten it. Post mortem examination of these hens showed in each case a well developed ovary with eggs in the several stages of development. Both the bodies were covered with fat and in the abdomen there were masses of fat weighing in each case over 500 g. The fat was quite white, owing to the colourless diet of rice and dried skim milk. This fat will have been formed from the carbohydrate and protein of the food which contained only 0.5 % of fat.

As was to be expected from the work of Palmer and Kempster [1919] the yolk of the eggs was colourless. Such white yolks were not observed by them nor by us in the eggs of the birds of Groups I to III. The whiteness of the yolks was best seen after hard boiling; it was then difficult to distinguish between white and yolk. Colour of yolk and reproduction have thus no relationship. The yolk of one of the eggs was analysed and found to correspond with the normal:

	Water %	Ash %	Protein %	Fat %
White yolk	44.4	1.8	15.8	36.7
Normal yolk	47.1	2.0	15.5	33.3

The palatability of the eggs was not different from that of the ordinary egg with yellow yolk.

The chicks hatched from these white yolk eggs were almost pure white, instead of the usual yellowish colour.

The size of the eggs on the average was normal; they seldom exceeded 2 oz., in general they averaged about 1½ oz., or 50 g.

It might appear from these experiments that hatchability was largely connected with the A-vitamin content of the food. This is probably only one factor of that problem; it is much more likely that protein, B-vitamin and proper salts are also concerned.

Group VII. The birds of this group were made into a breeding pen on Apr. 14th, 1923 (see Part III). After one week on the former diet of white rice, dried skim milk, dried yeast and cod-liver oil, the diet was changed to the simplest possible. It was composed of 100 g. rice, 20 g. dried yeast, 0.25 g. of salt and 1 g. of quick lime, slaked with water before being added to the food. The quantity of dried yeast was chosen as 20 g. as it had previously been found that 8.6 % of marmite = 17.2 % of dried yeast was a sufficient addition for 90 g. of rice and 5 g. of fish meal. This diet had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
9.8 %	0.2 %	2.7 %	0.2 %	12.5 %	0.5 %	74.0 %

The lime was added as it was found, on analysis, that dried yeast contained only traces of calcium.

Cod-liver oil to supply A-vitamin was omitted from the food up to the end of June, but the birds were given cod-liver oil by hand at the rate of 1 cc. per day for periods of five to seven days. The cod-liver oil was not given to any of the birds until May 15th, *i.e.* until they had been on the new diet for three weeks, at which period several of the birds were unwell. It was then given to the cock and two hens. The effect was striking. The cock who had lost his voice, again began to crow and became very active. The hens which had ceased to lay once more began laying. The dosing of these birds with cod-liver oil was then stopped and two other hens were given the cod-liver oil. They also began to lay after the doses of oil. These remarkable observations require repetition on another set of birds. They were not continued at the time, as birds from another group were mature and were added to this group as a breeding group, and as it was desired to ascertain the egg production and hatchability on this simple diet. Cod-liver oil was added to the food at the rate of 2.5 cc. per 100 g. of rice. The composition was not materially changed except as regards the fat as seen from the following figures:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
9.6 %	0.2 %	2.7 %	0.2 %	12.3 %	2.5 %	72.4 %

with a protein to fat and carbohydrate ratio of 1 : 6.1.

The results of this diet as regards fertility and hatchability of the eggs will not be known for some time.

At the time of change to this diet on July 1st the birds had been kept without C-vitamin for six months. The eggs laid between Apr. 29th and June 30th were tested for fertility and hatchability. The first eggs up to May 8th were all unfertile. Two eggs laid in June were put under a broody hen and both hatched out and both chicks were thriving well in August. The other eggs were incubated. They were mostly fertile and had well developed chicks; the ill success in hatching was probably due to the incubator being

not specially adapted for hatching eggs. C-vitamin thus appears to be not needed by the chicken at any period of its life.

Pigeons.

The two pairs of pigeons (see Part III) previously kept on a rice and fish meal diet for the comparison of marmite and cerema were placed, on Apr. 25th, 1922, on a diet consisting of 90 g. oatmeal, and 15 g. fish meal (1). This diet had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
8.6 %	0.5 %	4.3 %	0.9 %	18.9 %	7.7 %	59.5 %

B-vitamin was added to the extent of 0.75 g. of marmite for the red pair, and 1.5 g. of cerema for the blue pair. Cod-liver oil was not added, as it was probable that sufficient A-vitamin was present in the fish meal. The quantity of cerema was reduced to 0.75 g. 17 days after the start so that both pairs had then the same diet. C-vitamin was omitted.

The daily food consumption was on the average 45 g. oatmeal and 7.5 g. fish meal for each pair. It was greater during the periods of rearing young birds.

Both pairs of birds laid eggs every month during the breeding season, but except once in 1922 and once up to June 1923 no young lived longer than 10 to 14 days. The data of the eggs and rearing is given in the table. On account of the failure to rear the young birds, the diet was slightly altered:

(1) The marmite was omitted from the food of the red pair on June 18th, 1922; the cerema from that of the blue pair on July 13th, 1922.

(2) The fish meal was reduced from 15 to 10 g. on June 28th and to 5 g. on Aug. 1st for both pairs.

This diet consisting only of 90 g. oatmeal and 5 g. fish meal (1) was continued without alteration until June 10th, 1923. Its composition was

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
7.9 %	0.2 %	2.7 %	0.9 %	14.5 %	8.2 %	65.8 %

with a protein to fat and carbohydrate ratio of 1 : 5.1.

The alterations were made with the idea of diminishing the frequency of the egg laying. The failure to rear was possibly due to the desire of the hen bird to lay, as eggs appeared a few days after the young were apparently deserted. The alterations made no difference; one young bird was raised by the red pair from the last eggs of the 1922 season. The red pair again raised one young bird early in the 1923 season; this bird had crooked feet and was chloroformed. At the same time the observation was made that the parent birds did not actually desert their young. The cause of the failure was probably in connection with the diet; it is being sought for by alteration in B- and A-vitamin content. This has consisted in the addition of 1.5 g. of marmite to the food of the red pair and of the addition of 1 cc. of cod-liver oil to that of the blue pair per 90 g. of oatmeal. The effect has been the rearing of another

pair of young by the red pair, but the continued failure of the blue pair. Further experiments on this finding must await the next breeding season.

The maintenance of the two pairs in health for one year and two months, with the production of fertile eggs and at any rate three perfect young birds, is sufficient evidence that pigeons never need the inclusion of C-vitamin in their diet.

An unfortunate accident happened on Nov. 15th, 1922. The two blue birds escaped during the cleaning of their cage. The hen returned the same evening but the cock was lost. Luckily the single red pigeon which had been reared was a cock and he was now paired with the blue hen. All the data of the blue pair since Nov. 20th, 1922 refer to this red cock and blue hen. The red cock had been reared entirely without C-vitamin and the eggs produced were fertile (see table).

Table of egg production by pigeons.

Red pair				Blue pair			
Eggs laid on	Weights g.	Hatched	Result	Eggs laid on	Weights g.	Hatched	Result
15. v. 22	20.4	—	Unfertile	25. v. 22	19.0	11. vi. 22	Both squabs
16. v. 22	22.3	3. vi. 22	Squab died	26. v. 22	19.0	12. vi. 22	died
15. vi. 22	23.1	4. vii. 22	Squab died	24. vi. 22	19.0	12. vii. 22	Both squabs
	—	—	—	26. vi. 22	19.5	13. vii. 22	died
18. vii. 22	23.2	5. viii. 22	Squab died	25. vii. 22	19.0	11. viii. 22	Both squabs
19. vii. 22	24.7	5. viii. 22	„	26. vii. 22	20.0	12. viii. 22	died
23. viii. 22	22.2	9. ix. 22	Reared	25. viii. 22	19.2	11. ix. 22	Both squabs
24. viii. 22	24.0	9. ix. 22	Died	26. viii. 22	21.0	12. ix. 22	died
				27. ix. 22	18.0	14. x. 22	Both squabs
				29. ix. 22	19.4	15. x. 22	died
9. iii. 23	22.0	Eggs deserted, addled		6. ii. 23	19.5	Unfertile	—
11. iii. 23	23.0	27. iv. 23	—	8. ii. 23	18.0	„	—
8. iv. 23	22.5	Reared, crooked feet		24. iii. 23	20.5	7. iv. 23	Both squabs
10. iv. 23	23.5	—	Squab died	26. iii. 23	20.5	7. iv. 23	died
				18. iv. 23	18.5	6. v. 23	Both squabs
				20. iv. 23	19.5	7. v. 23	died
12. v. 23	23.0	29. v. 23	Both squabs	15. v. 23	19.0	3. vi. 23	Squab died
13. v. 23	?	30. v. 23	died	16. v. 23	Broken	—	—
13. vi. 23	23.0	2. vii. 23	Reared	11. vi. 23	20.0	29. vi. 23	Deserted
15. vi. 23	23.0	2. vii. 23	„	12. vi. 23	20.0	29. vi. 23	„

The last two eggs were laid after changing the diets, by adding marmite to that of the red pair, cod-liver oil to that of the blue pair.

The weights of the birds remained almost constant. Those of the blue pair rose at first after the recovery from the rice diet. The reduction after Jan. 1923 may be connected with the breeding season. The record was:

	Red cock	Red hen	Blue cock	Blue hen
	g.	g.	g.	g.
25. iv. 22	434	455	370	247
3. viii. 22	463	475	482	406
8. xi. 22	525	560	560	450
			Young red cock on	
			8. xi. 22:—430 g.	
8. i. 23	545	620	470	445
23. iii. 23	487	542	435	420
20. iv. 23	475	505	405	405

Other birds.

Day-old ducks, geese, turkeys, guinea-fowl, and pheasants have been used for ascertaining their B-vitamin requirements (see Part V, p. 794). They were all given a diet consisting of oatmeal and fish meal to which marmite and cod-liver oil were added. C-vitamin was not included.

Several losses amongst all the groups occurred, due to insufficient B-vitamin, but except in the case of turkeys, several birds were raised to maturity. One turkey was kept for 10 weeks. The other birds were kept for 12 to 16 weeks.

C-vitamin thus appears to be unnecessary for these birds.

SUMMARY.

1. Pigeons have been kept for 15 months and have laid eggs and reared young on a diet without C-vitamin.

2. Chickens have been kept from the day old stage to maturity without C-vitamin. They have laid eggs and a few young chicks have been hatched from the eggs.

3. Ducks, geese, turkeys, guinea-fowl and pheasants have been reared from the day old stage for periods varying from 12 to 16 weeks without C-vitamin.

4. C-vitamin does not appear necessary in the food of domestic and farm birds.

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XCVII. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART V. COMPARATIVE B-VITAMIN REQUIREMENTS OF PIGEONS, CHICKENS AND OTHER BIRDS.

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It is very tempting to think that the food requirements of all species of birds are alike. Sugiura and Benedict [1923] from their experiments with pigeons have been led to generalise that very little A-vitamin is needed in *avian* nutrition. Emmett and Peacock [1923] do not agree with this generalisation and have found that chickens require more A-vitamin than pigeons. The experience of poultry keepers is also against any idea that all birds have the same requirements. It is held by them that turkeys and pheasants at any rate are much more difficult to rear than chickens. Our own data with reference to pigeons and chickens on diets of oatmeal [Part I, 1922] and rice [Part III, 1923] indicate that the B-vitamin requirements of chickens are greater than those of pigeons. It is possible that the difficulties of rearing turkeys and other farm birds may be partly connected with the B-vitamin needs. Some preliminary experiments have therefore been undertaken to study this question. They have indicated that B-vitamin is an important factor in the difference between the ease of raising of chickens and other birds. Further experiments cannot be made till the next season. The present incomplete data are now put forward as they may be of some little value to the poultry keeper.

EXPERIMENTAL.

Oatmeal and rice had been used as the cereal in the previous experiments with chicks and pigeons. As source of protein the chicks had generally been given dried skim milk and the pigeons fishmeal.

With oatmeal, pigeons had been kept for 15 months without any addition of marmite as source of extra B-vitamin. Chicks had an addition of 0.75 g. of marmite per 90 g. of oatmeal.

With rice, at the time these experiments were started, both chicks and pigeons had an addition of 3 g. marmite per 90 g. rice. Dried skim milk had probably considerably more B-vitamin in it than fishmeal.

No definite conclusion could be drawn from these data, but it was likely that the B-vitamin requirements of chickens were greater than those of pigeons.

The pigeons were already on a diet of oatmeal (90 g.) and fishmeal (5 g.). To avoid changing their diet, and as chickens had previously been kept on oatmeal the pigeon diet was chosen for the comparative experiments.

The mixture of oatmeal and fishmeal had the following composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
7.9 %	0.2 %	2.7 %	0.9 %	14.5 %	8.2 %	65.8 %

with a protein to fat and carbohydrate ratio of 1 : 5.1.

The amount of ash was suitable, but the A-vitamin content was probably low. In the earlier experiments, no addition of cod-liver oil was made to supply A-vitamin, but later first 0.5 cc., then 1.0 cc., was added per 90 g. of oatmeal, in order to avoid complications on this account.

The experiments were not made in the order described but as follows: chicks, ducks, geese, ducks, guinea fowl, chicks, turkeys, pheasants.

Pigeons. The pigeons were kept on the same diet as described in Part IV. They were not able to rear their young without an addition of extra B-vitamin. Further evidence on this finding must await the next breeding season.

Chickens. Group X. It was not expected that chicks could be reared on oatmeal and fishmeal without the addition of marmite, but since the experiment had not been made it was necessary to establish the supposition, by allowing the birds to die if the food was not suitable.

The experiment was started on Apr. 7th, 1923, with ten day-old Light Sussex chicks.

One bird was unwell on arrival and died the next day. A second bird became ill on Apr. 9th, and died on the 8th day. All the birds showed ruffled feathers on the 12th day. A third bird was "off its legs" on the 17th day and died the following day. A fourth and fifth died on the 20th day. A sixth and seventh were ill on the 30th and 32nd days, and died on the 36th day. An eighth died on the 37th day. A ninth on the 39th day and the last on the 52nd day.

Post mortem examinations were made on the birds. In some cases nothing abnormal could be discovered. In others, the intestines were found full of undigested food. The chicks evidently suffered from constipation and died from absorption of toxic products formed by putrefaction in the gut. In two cases, a small swelling of the gut wall was found with a possible perforation. Chicks cannot thus be reared on a diet which suffices for adult pigeons.

Group XI. To prove that extra B-vitamin is needed by chicks on the oatmeal and fishmeal diet of pigeons, an addition of 1.5 g. of marmite per 90 g. of oatmeal was made. At the same time extra A-vitamin was included as cod-liver oil at the rate of 1 cc. per 90 g. of oatmeal.

The group consisted of 24 Black Leghorn day-old chicks and the experiment was started on June 1st and concluded on Aug. 17th, 1923 (11 weeks). One bird died on the second day. An unabsorbed yolk sac was found on post mortem examination. The remaining 23 birds were reared without any loss. Throughout the time of experiment the birds looked perfectly well and never showed signs of illness. The cockerels of the group began crowing in the seventh and eighth weeks. The best proof of their health is given by their steady rate of growth:

Average weekly weights.

	June 1	June 8	June 15	June 22	June 29	July 6
	g.	g.	g.	g.	g.	g.
	34.4	58.0	78	130	182	270
Increase	—	24	20	52	52	88
	July 13	July 20	July 27	Aug. 3	Aug. 10	Aug. 17
	g.	g.	g.	g.	g.	g.
	314	411	502	607	737	837
Increase	44	97	91	105	120	100

On the above diet the addition of 1.5 g. of marmite or 0.8 % of the food is sufficient for the health and growth of chicks. It is possible that 0.75 g. would be enough, but the more delicate birds would probably be lost.

Ducks. I. Aylesbury. The experiment was started on Apr. 12th, 1923, with four day-old Aylesbury ducklings.

During the first week there was an average gain in weight of 28 g.

One duck died during the second week; the others gained 21 g. Two other ducks died during the third week. The last duck died on the 30th day.

The pigeon diet was therefore not suitable.

II. White Runner. A group of 12 day-old White Runner ducklings was started on the oatmeal and fishmeal diet with an addition of 0.75 g. of marmite on Apr. 30th, 1923. Most of the ducks were shaky on their legs on the fourth day and seemed to have little appetite; they seemed better on the fifth day.

On the sixth day, five of them showed the typical symptoms of polyneuritis with the head bent back as is seen with pigeons. They were dosed with 2 cc. of 2 % marmite. Three died, but the other two were better; they died, however, on the next day.

0.75 g. marmite was obviously insufficient. It was raised to 2.25 g.

As no extra A-vitamin was present in the food, 0.5 cc. of cod-liver oil was included on the 10th day.

With the extra marmite the ducks flourished for the next two weeks. As it was desired to find the lowest amount of marmite necessary, the quantity was reduced on the 22nd day to 1.5 g. per 90 g. of oatmeal.

After this reduction another duck died on the 30th day; the gut was found full of food and perforated by a piece of undigested oatmeal.

During the sixth week, two ducks were noticed with a sort of contracted neck; one of them was better the next day; the other recovered after receiving several doses of 1 g. marmite dissolved in about 10 cc. of water.

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As the ducks were getting ill on 1.5 g. additional marmite, the amount was obviously insufficient and it was raised to 2.25 g. on the 40th day. On the 47th day the cod-liver oil was raised to 1 cc.

After these additions all the ducks were kept in good health till the experiment was ended on 17th Aug. The duck which had suffered from the contracted neck and recovered after the doses of marmite always remained smaller than the others.

Six out of twelve ducks were reared with the addition of 2.25 g. of marmite, the other six died with a smaller quantity per 90 g. oatmeal.

The average weekly weights show that the final amount was sufficient:

	Apr. 30	May 7	May 14	May 21	May 28	June 4	June 11	June 18
	g.	g.	g.	g.	g.	g.	g.	g.
	36.3	56.8	127.7	265	411	690	817	1036
Increase	—	20.5	70.9	137	146	288	118	219

	June 25	July 2	July 9	July 16	July 23	July 30	Aug. 6	Aug. 13
	g.	g.	g.	g.	g.	g.	g.	g.
	1197	1305	1473	1454	1543	1629	1578	1603
Increase	161	108	168	- 19	89	86	- 51	25

The two occasions on which a decrease in weight was observed were during very hot weather, not much food being eaten.

Geese. This experiment was started with 13 day-old goslings on Apr. 26th, and concluded on Aug. 17th, 1923 (16 weeks).

It had been found that chicks and ducklings could not be reared on the oatmeal and fishmeal diet without additional B-vitamin. It was not likely that it would be suitable for day-old goslings, so 0.75 g. of marmite was included at the start.

The birds were rather unsteady on their feet on arrival, but looked better after a few days, though they were very quiet. They gained on the average 51 g. in weight in the first week.

Two died on the eighth day. No cause of death could be discovered on post mortem examination.

The marmite was therefore raised to 1.5 g. per 90 g. oatmeal.

Two more died on the ninth day. Since the others looked ill, they were given a dose of 4 cc. of 2 % marmite for the remainder of the second week. Six more however died. Post mortem examination showed the gut filled with food, largely undigested; in one case there was perforation of the intestine. Death was probably due to absorption of toxic products from the stagnated food in the gut.

At the beginning of the third week, 0.5 cc. of cod-liver oil was put into the food. It was increased to 1 cc. per 90 g. oatmeal at the seventh week to conform with the food of the other kinds of birds.

The three remaining goslings survived the fourth week though their condition was not satisfactory. One died at the beginning of the fifth week. Marmite was then raised to 2.25 g. per 90 g. oatmeal.

After this addition the last two geese were kept till Aug. 17th without any illness. Their final weights averaged 5835 g.

2.25 g. marmite is thus needed by geese on the oatmeal and fishmeal diet.

Guinea Fowl. Five day-old and six rather older guinea fowl were used for this experiment. It was started on May 18th and concluded on Aug. 17th (13 weeks).

From the first day an addition of 1.5 g. marmite and 0.5 cc. of cod-liver oil was made. To conform with the other groups the cod-liver oil was increased to 1 cc. per 90 g. of oatmeal at the beginning of the fourth week. Two birds died during the first week; one of them had its caeca full of food blocking the passage through the gut. The others at this period had very little appetite. On this account the marmite was raised to 2.25 g. per 90 g. oatmeal (May 25).

A third died during the second week, and a fourth during the third.

No further loss occurred. The remaining seven were kept without any signs of illness. Their feathering was perhaps not as good as is seen normally. The growth of the birds is seen by their average weekly weights:

May 18	34 g.	June 1	92 g.	July 6	463 g.	Aug. 3	830 g.
" 25	57	" 8	156	" 13	551	" 10	966
		" 15	212	" 20	619	" 17	1028
		" 22	295	" 27	724		
		" 29	359				

Again, 2.25 g. is the required addition of marmite to the diet.

Turkeys. Ten day-old turkey poults were started on the diet with 1.5 g. marmite and 1 cc. of cod-liver oil on May 26th. One bird was kept till Aug. 5th, 1923. The birds did not like the diet and ate very little. At the end of the first week six had died. The others only gained an average of 6 g. Marmite was at once raised to 2.25 g. per 90 g. of oatmeal.

With this increase, the remaining four gained 15 g. during the second week. An unfortunate accident then occurred in which three of them were lost by fire in the foster mother. The last bird grew steadily each week, but in the tenth week it met with some accident in which its legs were damaged and it was chloroformed.

It appears thus that 2.25 g. of marmite is just sufficient for rearing turkeys on the oatmeal and fishmeal diet.

The bird weighed 560 g. at the end of the tenth week.

Pheasants. These birds were most kindly supplied for the experiment by Mr J. Q. Rowett. The experiment was started on June 4th and terminated on Aug. 27th, 1923. 1.5 g. of marmite and 1 cc. cod-liver oil were added to the food mixture at the start.

One bird died during the second week; no definite symptoms could be found on post mortem examination. Another broke its leg and was chloroformed. During the third week three died. These were found to have enlarged caeca causing stoppage in the gut.

The marmite was raised to 2.25 g. after these losses. Except for one more loss in the fifth week, the others were reared for 12 weeks. Unfortunately

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one of them damaged its leg and had to be chloroformed. Though they lived and grew quite well, it was very noticeable that the food was not entirely suitable; their sizes were very unequal and their feathering was very poor. The larger birds were continually pecking at new feathers which formed on the smaller ones.

Their rate of growth was slow as seen from their weekly weights:

June 4	18.8 g.	July 2	72 g.	Aug. 6	240 g.
" 11	25	" 9	96	" 13	295
" 18	35	" 16	132	" 20	356
" 25	49	" 23	174	" 27	422
		" 30	200		

GENERAL REMARKS.

Except adult pigeons and chicks, all the birds have needed an addition of 2.25 g. of marmite per 90 g. of oatmeal for their successful rearing. Chicks required 1.5 g.

This quantity is the minimum. Better results would undoubtedly have been secured with an addition of 3 g. per 90 g. oatmeal. 1 cc. of cod-liver oil per 90 g. oatmeal is sufficient.

The diet was specially chosen with only 5 g. of fishmeal. An increase to 10 g. would be likely to give very satisfactory growth. As pointed out in Part III, a change of cereal might not lead to a successful result since the quantity of B-vitamin in cereals is not yet known.

A diet upon which adult pigeons can exist is certainly inadequate for all other farm birds. It is curious that chickens have a smaller B-vitamin requirement than the others. It is possible that they are able to digest oatmeal better and thus obtain the whole of the B-vitamin present in it, whereas some of this B-vitamin is not assimilated by the other birds. The difference is not great.

SUMMARY.

Pigeons on 90 g. oatmeal and 5 g. fishmeal do not require any extra B-vitamin. Chickens on the same diet need an addition of 1.5 g. marmite per 90 g. of oatmeal.

Ducks, geese, guinea fowl, turkeys, pheasants, need an addition of 2.25 g. of marmite to supply extra B-vitamin.

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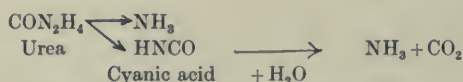
XCVIII. UREASE. PART II. THE MECHANISM OF THE ZYMOlysis OF UREA.

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(Received September 5th, 1923.)

IN a previous communication [Fearon, 1923] an account was given of the detection and estimation of cyanic acid in solutions of urea undergoing zymolysis, and it was suggested that cyanic acid and ammonia are the intermediate stages in the enzymic decomposition of urea:



Since this paper was submitted for publication, a paper has appeared on the mechanism of urease by Mack and Villars [1923]. In this paper the authors agree with the present writer in accepting the Werner formula for urea. They have detected and estimated cyanate in solutions of urea undergoing zymolysis, and also have shown that the cyanate rises to a maximum and falls again towards the end of the zymolysis. Their maximum values expressed as percentage cyanic acid agree closely with several maximum values obtained by the present writer (0.003 %).

On the other hand, they arrive at the conclusion, by a process of elimination, that the transformation



is the stage catalysed by the enzyme, and they do not appear to consider cyanic acid as a possible intermediate product in the zymolysis.

This conclusion is based on indirect evidence; carbamic acid or carbamates have not been shown to be present in the urea/urease system. The work of Yamasaki [1918] is inconclusive in this respect, since the experimental method he adopts will not discriminate between carbamates and cyanates.

The method employed by Mack and Villars in estimating cyanic acid consists in precipitating the samples with excess of silver nitrate, washing the precipitate free from ammonia, digesting with dilute nitric acid to convert the cyanate into ammonium nitrate, and estimating the ammonia so formed by nesslerisation.

When silver nitrate is added in excess to a mixture of ammonium carbonate, carbamate, and cyanate in solution, a precipitate is thrown down consisting of the silver salts of the three acids. If the solution be now set to p_{H} 5 by means of dilute nitric acid the carbonate and carbamate dissolve leaving the silver cyanate. This is the basis of the method adopted by the present writer in estimating cyanic acid [1923].

The method of Mack and Villars is open to the objection that any carbamate present would be included in their result as cyanate; this defect can be overcome by setting the solutions to a suitable p_{H} before filtration. Thus modified, their method is much more convenient than that formerly adopted by the present writer, especially now that a preparation of urease containing a minimum amount of protein is available (*urease Dunning*).

To determine the exact significance of the Mack and Villars results, and to obtain information as to the part played by carbamate in the urea/urease system, the cyanate and carbamate in solutions of urea undergoing zymolysis were determined simultaneously.

Estimation of the Carbamate and Cyanate in the Urea/Urease System.

Solutions of pure urea and urease (*Dunning*) of such proportions as to produce 0.1 *M* urea and 0.1 % urease were mixed and incubated at 20°. At definite intervals duplicate samples of 50 cc. were withdrawn and treated with excess of $N/1$ AgNO_3 . One sample was filtered at once, this gave the carbamate and cyanate residue. The other sample was set to p_{H} 5 by means of 0.6 *N* HNO_3 , using methyl-red and a comparator, and then filtered, this gave the cyanate residue only. The residues were washed until free from ammonia, and then were digested on a water-bath with 20 cc. of 2 % HNO_3 . After cooling, they were treated with 15 cc. of saturated K_2CO_3 , diluted to 50 cc. and the ammonia determined by aspiration into 100 cc. of $N/500$ HCl , and subsequent nesslerisation. The results are expressed as nitrogen derived from both carbamate and cyanate and from cyanate alone in the 50 cc. samples. The difference between these values represents carbamate alone. By titration of samples from time to time with 0.6 *N* HNO_3 , using methyl-red, the progress of the zymolysis was roughly determined.

Table I.

Time in minutes	Carbamate and cyanate N in 50 cc. $\times 10^4$ g.	Cyanate N in 50 cc. $\times 10^4$ g.	Cyanic acid %	Urea % decomposed
0	0	0	0	0
30	1.1	1.0	0.0006	18.6
60	2.3	2.5	0.0015	—
130	5.2	5.0	0.0031	52
240	2.0	2.3	0.0014	91
1320	0.1	?	0	99.9
Substrate 0.1 M urea.			Temperature 20°.	
Enzyme 0.1 % urease Dunning.				

From these results it will be seen that the cyanate N and the carbamate N plus carbamate N agree; and consequently there is no indication of the presence of carbamate at any stage during the zymolysis of urea. The results of Mack and Villars may be taken as representing cyanate, as will be seen from a comparison of their figures with the above table.

Table II. (*From the data of Mack and Villars [1923]*)

Time in minutes	N in 50 cc. $\times 10^4$ g.	Cyanic acid %
5	2.33	0.0014
10	2.75	0.0016
20	3.46	0.0021
80	5.59	0.0034
165	3.99	0.0024
Substrate	0.1 M urea.	Temperature 25°.
Enzyme	0.1 % urease.	

Mack and Villars conclude that the cyanate in the urea/urease system is due to the spontaneous dissociation of the urea, and is uninfluenced by the presence of the enzyme.

That this is not so was demonstrated by a control solution of urea alone, which showed no indication of cyanate formation during the time of the experiment, nor for several days unless it had become infected.

The Urea-Ammonium Cyanate Equilibrium.

Urea in aqueous solution when kept *sterile* appears to be stable at ordinary temperatures for an indefinite period. Werner [1918] has found $N/2$ solutions of urea in toluene water free from cyanate or carbonate after nine months. On the other hand, a similar solution of urea in plain water showed signs of decomposition after 14 days at room temperature. A 5 % solution of urea kept by the author under ordinary laboratory conditions for one year showed on analysis a conversion of 0.2 % of the urea into ammonium carbonate. A mould with urealytic properties was found to have developed in the liquid. The solution contained no cyanate; the activities of the mould had presumably been inhibited by the increasing alkalinity of the solution, and the cyanate had been hydrolysed to carbonate.

Werner explains the stability of urea in sterile solutions below 40° as being due to the fact that it is not dissociated into ammonia and cyanic acid under such conditions. If, however, these solutions are heated to about 60° dissociation occurs with production of cyanic acid and ammonia. This dissociation has been very fully investigated by Walker and Hambly [1895], who have shown that, after one hour at 100°, 4 % of a $N/10$ urea solution had come into an equilibrium with ammonium cyanate produced by the transformation of the urea.

The equilibrium concentration for 39° found by incubating $N/10$ solutions containing 95 % urea and 5 % ammonium cyanate, is open to several experimental objections. Part of the cyanate is converted into urea and part is hydrolysed to ammonium carbonate.

The urea-ammonium cyanate equilibrium is not stable as the cyanate is gradually hydrolysed to ammonium carbonate until all the urea is removed from solution by this double process of dissociation and hydrolysis.

This change is slow if the ammonia be prevented from leaving the system. Fawsitt [1902] found that 450 hours were required to bring about the de-

composition of 98.9 % of the urea originally present in a semi-normal solution kept at 99° in a sealed tube.

The Formation of Ammonium Carbamate from Urea.

The direct formation of ammonium carbamate by the hydration of urea has not been demonstrated experimentally. Its possible occurrence is open to doubt. The various syntheses of urea from ammonium carbonate and from ammonium carbamate have been shown by Werner to involve an intermediate formation of cyanic acid from the dehydration of carbamic acid. It is hoped to discuss the very interesting subject of the enzyme synthesis of urea from ammonium carbonate and carbamate claimed by several investigators [Barendrecht, 1919; Mack and Villars, 1923; Kay, 1923] in a subsequent paper.

Cyanic Acid as the Intermediate Stage in the Urea/Urease System.

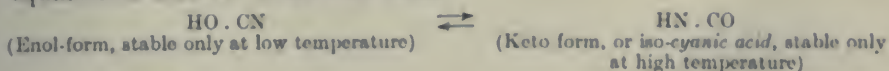
Before a compound can be considered as a possible intermediate stage in a catalysed reaction it must be shown to conform to the requirements of Ostwald's law of successive reactions [1902]. The intermediate reaction must take place more rapidly than the direct reaction under the same conditions. The concentration of such a compound will depend on its stability. As Hopkins has observed: "the degree to which a substance accumulates is itself no measure of its metabolic importance, no proof as to whether it is on some main-line of change, or a stage in a quantitatively unimportant chemical by-path" [Bayliss, 1917]. Cyanic acid in aqueous solution is unstable, and the degree to which it accumulates will depend on the difference between the rate of formation and the rate of removal. At the start of the reaction the cyanic acid gradually reaches a maximal concentration depending on the temperature and concentration of the enzyme, but independent of the concentration of substrate, provided that there is excess of substrate.

Working with low concentrations of enzyme preparation (0.1 to 0.2 %) and substrate (0.1 to 0.5 *M* urea) at temperatures of 5 to 20° the maximum concentration of cyanic acid is 3-4 mg. %. The figures obtained by the method of Mack and Villars, adopted in the present paper, are probably more accurate than those obtained by the use of collodion sacs described in the previous paper [Fearon, 1923], owing to the diffusion being retarded by the sac.

Towards the end of the zymolysis the rate of formation of cyanic acid begins to decrease owing to decrease of substrate and accumulation of ammonia in the system, which retards the hydrolysis of cyanic acid, as well as the dissociation of the substrate. Consequently, the removal of cyanic acid decreases in rate also towards the close of the reaction.

The Hydrolysis of Cyanic Acid.

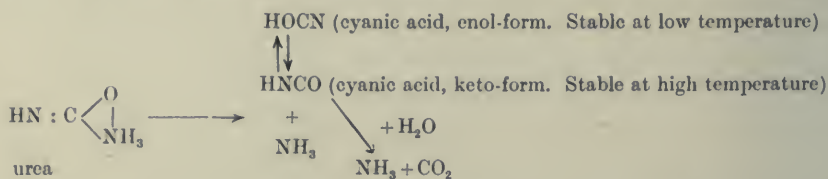
In the liquid state below zero, and in aqueous solutions, cyanic acid is an equilibrium mixture of the two forms:



Liquid cyanic acid, below zero, contains about 60 % HOCN and 40 % HNCO . Rise of temperature leads to increase in HNCO , and fall of temperature increases the HOCN [Werner and Fearon, 1920]. Union of enol-cyanic acid with ammonia produces ammonium cyanate.

Union of keto-acid with ammonia produces urea.

When urea in solution undergoes dissociation, ammonia and cyanic acid in the keto-form are produced. Keto-cyanic acid is less stable at low temperatures than the enol-form of the acid, accordingly, some of it is transformed into enol-cyanic acid and some of it is hydrolysed to ammonia and carbon dioxide. The result may be represented as follows:



Owing to the existence of these two forms, the hydrolysis of cyanic acid in aqueous solution is complicated, and involves three distinct primary changes [Werner and Fearon, 1920]:

- (1) $\text{HOCN} \rightleftharpoons \text{HNCO} + \text{H}_2\text{O} = \text{CO}_2 + \text{NH}_3$.
- (2) $\text{HOCN} + \text{NH}_3 = \text{NH}_4 \cdot \text{OCN}$ (ammonium cyanate).
- (3) $\text{HN : CO} + \text{NH}_3 = \text{HN} \cdot \text{CONH}_3$ (urea).

At the outset of the reaction the keto-cyanic acid is hydrolysed with the production of CO_2 and NH_3 . In 15 minutes 15 % of a $N/4$ solution of cyanic acid at 0° was converted into CO_2 and NH_3 . As the ammonia gradually begins to accumulate in the solution it unites with the keto-acid to form urea, and the hydrolytic process gradually slows down.

In the presence of urease the hydrolysis of cyanic acid is considerably modified. First, since the urea molecule is decomposed at a comparatively low temperature (0° to 55°) most of the cyanic acid is liberated into solution in its less stable form and is more readily hydrolysed than if liberated from a normal cyanate. Then, urease is able to combine with ammonia, reducing its concentration in the liquid phase of the system. Also, the urea formed synthetically during the late stages of the hydrolysis of the cyanic acid is in turn broken down by the enzyme.

All these factors will increase the amount of free keto-cyanic acid in the solution, which is the form in which the acid is most readily attacked by water.

The Mechanism of Urease Action.

As the present paper is chiefly concerned with the chemistry of the zymolysis of urea the interpretation of the data obtained from the study of the rates of zymolysis under varying conditions will not be discussed. The

most comprehensive account to date will be found in the work of Lovgren [1921], which includes an exhaustive bibliography of 212 references. The problem at issue can, however, be restated in more precise terms in view of the detection of cyanic acid in the urea/urease system. The urealytic property of the enzyme is due to its power of dissociating urea in solutions at ordinary temperatures, from 0° to 60° (above which the enzyme is rapidly destroyed).

The Adsorption Stage in the Urea/Urease System.

With the exception of Barendrecht [1919] and Yamasaki [1918], there is general agreement amongst the investigators of the kinetics of the urea/urease system that the first stage in the zymolysis is a union between the substrate and the enzyme. The precise nature of this union has not yet been determined. Armstrong, Benjamin and Horton [1913], Bayliss [1915, 1918] have come to the conclusion that urease acts by surface condensation or adsorption. Van Slyke and Cullen [1914] consider that the change is due to a chemical combination of enzyme and substrate, followed by a decomposition of the intermediate product according to the laws of chemical reactions deduced from mass action.

In support of the adsorption theory, Bayliss [1915] has described some simple and convincing experiments in which he shows that urease is able to act in a medium in which it is quite insoluble. Working with solutions of 4 % urea in 80 % to 89.3 % alcohol he found that urease was able to decompose from 7.8 % to 1.8 % of the substrate although the enzyme was quite insoluble in the medium.

The Action of Urease in Absolute Alcohol.

The determination of the degree of adsorption of urea by urease was attempted by extending the experiments of Bayliss to solutions of urea in absolute alcohol.

A preparation of soy-urease freed from fat by ether extraction in a Soxhlet extractor, was extracted at 40° with absolute alcohol until the filtrate no longer gave an opalescence on dilution with water and addition of silver nitrate. 50 g. of this preparation, which was very active, were added to 50 cc. of a saturated solution of pure urea in absolute alcohol at 18°. The mixture was incubated in a closed flask at 18°, and frequently shaken. A similar flask containing a saturated alcoholic solution of urea was kept under the same conditions as a control. The urea in each solution was determined from time to time by drawing off 2 cc. samples and determining the urea gravimetrically after evaporation of the alcohol on a water-bath. Equilibrium was reached after ten days.

Table III.

	%
Urea in control solution	4.290
„ in presence of enzyme	3.475
„ removed from solution	0.815
„ adsorption	19.0 of urea present

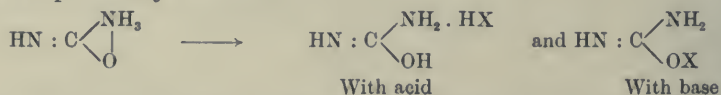
On filtering off the enzyme, the filtrate was found to contain no ammonia, but on dilution with water and after addition of silver nitrate gave a white precipitate soluble in dilute nitric acid, and responding to the test for cyanate by hydrolysis and nesslerisation, as described in Part I of the present communication [1923]. This was not observed in the control solution of urea, or in suspensions of the enzyme in absolute alcohol. The residue from the experiment was still active, and appeared unchanged.

This experiment supports the theory that adsorption is the preliminary stage in the zymolysis of urea. It also indicates that adsorption alone is inadequate to explain the change. Only a very small fraction of the adsorbed urea can have been dissociated.

It may be possible to find an explanation in terms of general adsorption for cases of enzymes which accelerate reactions, such as the hydrolysis of esters, where increase of concentration of the reactants in the surface layer will bring about increase of reaction-rate in accordance with the law of mass action. This subject has been recently reviewed by Bayliss [1919]. But in the case of enzymes such as urease, which initiate, or appear to initiate, reactions, general adsorption is not sufficient to explain the zymolysis, since a saturated solution of urea is quite as stable as a weak solution, in the absence of infection. Increase of concentration in the surface layer is by itself insufficient to bring about decomposition.

Selective Adsorption in the Urea/Urease System.

The adsorbent properties of urease, apart from its urealytic power, do not appear to have been investigated hitherto. Urea in neutral solution is present as a closed-ring compound. In this form it is not attacked by nitrous acid and does not give a condensation derivative with xanthhydrol [Werner, 1923]. The action of acids or alkalis is to open up the ring and form salts with the amphoterie *free* urea:



When the neutral urea molecule undergoes dissociation it gives rise to an alkaline component, ammonia, and an acid component, keto-cyanic acid. Acids or alkalis accelerate the decomposition of urea in aqueous solutions above the dissociation point by combining with either the alkaline or the acid component.

The behaviour of urease towards ammonia and cyanic acid was investigated in order to see if, in addition to its property of adsorbing urea, it showed any evidence of selective adsorption, or greater preference for one constituent of the system. Preliminary observations were made on the nature of the electric charge borne by urease in colloidal suspension.

Solutions of soy-urease, decanted from 5 % suspensions, and dialysed for a couple of hours against distilled water to remove excess of electrolytes,

were subject to a potential difference of 100 volts in a conductivity tube (Hardy's modification of Whetham's apparatus), giving a fall in potential gradient of 1 volt per cc.

The colloid showed an electro-negative charge. After 20 minutes at 15° the particles had accumulated in the region of the anode.

Control experiments with urease suspensions boiled for 10 minutes showed an almost complete disappearance of charge.

In the absence of more precise information as to the nature of the enzyme found in the urease preparations conclusions from experiments on impure preparations must be tentative. When an active preparation of urease free from foreign protein is available the work will be repeated.

The behaviour of soy-urease towards ammonia was then investigated. If the ammonia group of the neutral urea is adsorbed by the electro-negatively charged surface leaving the unstable cyanic acid in the outer zone of the Helmholtz "double layer," the rapid decomposition of the substrate should continue until the surface is saturated with adsorbed ammonia.

It is well known that ammonia retards the action of urease, as first shown by Armstrong and Horton [1912], but the fact that urease is able to adsorb ammonia has not been previously demonstrated.

The Adsorption of Ammonia by Urease.

Samples of soy-urease freed from fat were shaken up with solutions of ammonium sulphate of varying strength. After standing for 24 hours at room temperature, the mixtures were centrifuged and the ammonia in the supernatant liquid determined by the colorimeter.

The mixtures consisted of 2 g. soy-urease and 50 cc. standard ammonium sulphate (Folin). The average temperature was 18°.

Table IV.

	Before adsorption. Ammonia N in mg. %	After adsorption. Ammonia N in mg. %	Percentage adsorption
A.	28	16.6	40.8
B.	14	6.5	53.5
C.	7	0.0	100

A control solution of urease (4 %) in water alone did not liberate any ammonia after 24 hours.

The supernatant liquid from a 4 % solution of soy-urease in water alone, after standing for 24 hours before being centrifuged, contained no ammonia.

For the purposes of comparison, the ammonia-adsorbing property of a sample of permutit was determined under the same conditions.

The mixture consisted of 2 g. of washed permutit and 50 cc. ammonium sulphate solution. Time, 24 hours. Temperature 18°.

	Before adsorption. Ammonia N in mg. %	After adsorption. Ammonia N in mg. %	Percentage adsorption
	28	6.6	76.4

Permutit does not adsorb urea in aqueous solution.

Urease is not destroyed by the action of ammonia or ammonium salts at ordinary temperatures. Suspensions of soy-urease in $N/10$ NH_4OH after three months at 12° to 14° regained urealytic activity on washing with 2% acetic acid and with water. Even solutions of urease incubated with excess of 10 N ammonium carbonate and carbamate for 10 hours at 55° , in accordance with the method of Mack and Villars [1923], for showing the synthesis of urea, on removal of the ammonia were active towards urea, although a considerable amount of the enzyme material had been destroyed by the action of the strong alkali.

The action of urease on cyanic acid is difficult to investigate, since it is not possible to obtain the unstable form of the acid in solution apart from interfering substances. Mack and Villars have studied the action of urease on 0.1 M solutions of ammonium cyanate (made by mixing equal parts of 0.1 M solutions of NH_4Cl and KCNO) at 25° .

They find that urea is formed more readily from such solutions in the absence of urease than in its presence, which is as might be expected, because the concentration of ammonia in their experiments is not sufficient to inhibit completely the action of the enzyme.

Urease does not increase the rate of formation of *ammonium cyanate* from urea, and consequently need not necessarily catalyse the reverse reaction. The action of urease is to liberate the unstable form of cyanic acid from urea; this is rapidly hydrolysed until the progress of the reaction has resulted in the accumulation of ammonia sufficient both to retard the enzyme by forming an adsorption compound to the exclusion of the substrate and to retard the hydrolysis of the keto-cyanic acid. Ammonium cyanate can only appear at a late stage in the urea/urease system.

The Significance of Ammonia Adsorption in the Urea/Urease System.

Ammonia adsorption may in itself be sufficient to account for the dissociation of adsorbed urea, but it must be remembered that several other factors are concerned. The concentration of adsorbed substrate is difficult to determine, and until it is known, the ammonia concentration on the enzyme surface can only be inferred from kinetic data.

Urea adsorption by urease is a rapid process in aqueous solution, as was found by addition of soy-urease to solutions of urea just above freezing-point, and rapid separation again of the enzyme by alcoholic precipitation and centrifugalisation. The residue was then taken up with water, and incubated. The adsorbed urea underwent decomposition, thus affording a rough method of determining the extent and rate of adsorption.

Substrate, 10 cc. of 2 % urea. Temperature 0° .

Enzyme, 0.1 g. urease *Dunning*, in fine powder.

The mixture was shaken for 30 seconds, and then treated with 80 cc. absolute alcohol, which completely precipitated the enzyme. The mixture

was then rapidly separated by centrifugalisation, and the residue was incubated at 36°.

Ammonia formed = 19 mg. urea, or 9.5 % of substrate present.

By an extension of this method the rate of adsorption might be determined. The conclusions from qualitative experiments on the mechanism of enzyme action must be restricted owing to lack of knowledge as to the degree of purity of the various preparations, and the necessity for distinguishing between accidental and essential properties of the ferment.

Until urease is obtainable in a form of biochemical purity, corresponding to the *amylase* prepared by Sherman, an extension of the work outlined in the present paper does not appear to be profitable.

In addition to the effect of selective adsorption of ammonia it must be noted that adsorbed urea is probably subjected to a considerable mechanical pressure in the surface layer.

Williams [1920], working with charcoal, has calculated the surface pressure to be of the order of 50,000 atmospheres. Many of the instances of irreversible adsorption may be ascribed to compression effects, such as the coagulation of adsorbed proteins at ordinary temperatures, a change which in the absence of the adsorbent requires pressures of 17,000 to 20,000 atmospheres [Bridgman 1914].

The dissociation of urea gives rise to the very compressible base ammonia, hence it is to be expected, although it has not yet been demonstrated, that high pressures alone should bring about the dissociation of urea. Lewis and Burrows [1912] have found that the decomposition of urea in solution by heat is always accompanied by a diminution in volume. It has also been found that in the Haber ammonia process increase of pressure displaces the equilibrium so that more is formed of the component occupying the lesser volume, namely ammonia [Haber, 1914].

A Theory of the Mode of Action of Urease.

From qualitative data it is possible to construct a theory of the mode of action of urease, which will explain, amongst other things, the specific activity of the enzyme.

Urea is adsorbed from solution by urease and then dissociated into ammonia and cyanic acid in its unstable form. The ammonia is adsorbed by the enzyme, the cyanic acid remains in solution.

In aqueous solutions the cyanic acid is rapidly hydrolysed into ammonia and carbonic acid. The resulting ammonium acid carbonate is able to combine with another molecule of ammonia, and so will tend to reduce the concentration of adsorbed ammonia, thus freeing the enzyme.

For this reason, acids in low concentration accelerate the reaction by combining with the ammonia. Alkalies in low concentration retard the zymolysis by neutralising the carbonic acid, by fixing the cyanic acid as a much more stable cyanate, and also by being adsorbed themselves.

Carbonic acid being a weak acid, and its salts with ammonia being largely dissociated, a stage is reached in the urea/urease system when the enzyme is approaching saturation with ammonia, and the reaction rate steadily falls, depending on the diffusion of the end-products into the solvent. If at this stage the H-ion concentration of the solution be increased to its optimum range (p_H 6.8–7.0) by buffers or by aeration with carbon dioxide the reaction rate returns to its maximum, provided the substrate be in excess. Under such conditions the rate of the zymolysis approximates to linear [Armstrong and Horton, 1912]. Here, what is being measured is the rate of hydrolysis of free cyanic acid at an optimum H-ion concentration.

Acids in high concentration stop the zymolysis of urea completely; the action is probably due to the precipitation of the enzyme, either directly or associated with the globulin of the urease preparation.

In higher concentrations of acids some of the urease preparation goes into solution once more, but has no action on urea. This may be due to a reversal of the electric charge, but until the purification of urease has been accomplished the subject cannot be thoroughly investigated.

An additional, but probably less important, effect of acids is their action on the substrate itself, opening up the cyclic form of urea to produce the salt form which is not dissociated into cyanic acid and ammonia.

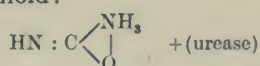
Reversibility of Action of Urease.

Since ammonia and carbon dioxide are not the primary products of the action of urease on urea, its synthesis from these substances is not an inevitable consequence of the general theory of enzyme action. Mack and Villars [1923] and Kay [1923] have undoubtedly obtained urea from the action of strong solutions of ammonium carbamate and carbonate on urease. However, it has not yet been demonstrated that this urea arises from an enzyme dehydration of the carbamate, and it appears unlikely, since Werner has shown that the classical Basarov synthesis [1868] of urea by the action of heat on ammonium carbamate involves the intermediate formation of cyanic acid, as first suggested by Mixter [1882].

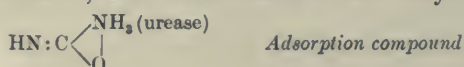
The results of an extended investigation following the lines of Mack and Villars will be described in a subsequent communication.

Summary of the Dissociation Theory of Urease Action.

(1) At the beginning of the reaction the urea/urease system consists of the non-electrolyte urea in true solution, and the enzyme urease in suspension as a negatively-charged colloid:



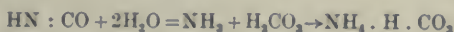
(2) *Stage of Adsorption* (occurs in alcoholic and aqueous solutions). Urea is withdrawn from solution, and concentrated on the enzyme surface:



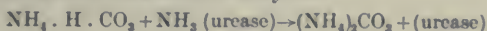
(3) *Stage of Dissociation.* Adsorbed urea is dissociated into keto-cyanic acid and ammonia, which is held by the enzyme:



(4) *Stage of Hydrolysis* (occurs in aqueous solutions only). The keto-cyanic acid is hydrolysed by the solvent, with the formation of ammonia and carbonic acid:



(5) The carbonic acid and acid carbonate combine with some of the adsorbed ammonia, and liberate the enzyme:



(6) The liberated urease combines with more urea, and so the cycle continues, until brought to rest by exhaustion of substrate or by accumulation of ammonia in sufficient concentration to inhibit stage (5). Stage (5), the breakdown of the adsorption compound, is the true characteristic of the urea/urease system (and probably all catalytic actions in heterogeneous systems). In the absence of stage (5) there can be no continuous zymolysis; the chemical changes would be linear, not cyclical.

CONCLUSIONS.

1. No evidence has been obtained of the occurrence of carbamic acid in the zymolysis of urea. Cyanic acid and ammonia appear to be the intermediate products of the action of urease on urea. Urease acts by dissociating its substrate.

2. Soy-urease is able to adsorb urea from alcoholic solution.

3. Soy-urease in aqueous solution carries an electro-negative charge, and is able to adsorb ammonia from aqueous solution.

4. A theory of the mechanism of urease is presented in outline. Urease combines with urea by adsorption. The adsorbed urea is dissociated into ammonia, which combines with the enzyme, and cyanic acid, which is hydrolysed by the solvent. The enzyme is then liberated from the ammonia compound.

5. It is suggested that urea dissociation is brought about by selective adsorption and also possibly by the pressure in the surface layer.

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XCIX. THE INFLUENCE OF SURFACE TENSION ON THE GROWTH OF BACTERIA.

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ONE of the factors which may be anticipated to affect the growth and metabolism of bacteria is surface tension.

The relation of this physical factor to other biological problems such as muscle action or the deposition of salts in plant cells [Macallum, 1911] has been the subject of much study; the relation to bacterial growth has received little attention.

If, however, one considers the results obtained with surface films by J. J. Thomson [1888] and others it is evident that the increased concentration of substances, such as peptone, at the air/liquid interface ought to have a pronounced effect on those organisms growing at the surface. This should especially be the case with micro-organisms like *C. diphtheriae* where experience has shown that efficient toxin production is associated with good pellicle formation.

In a study of certain factors affecting toxin production my attention was drawn to this aspect of the subject by the statements of practically every bacteriologist who has worked in this field as to the necessity of removing all but inevitable traces of fat from the medium before it is used. The fact that pellicle is also essential would appear to be connected with the particular conditions of the air/liquid interface.

In a paper published in 1919 Larson, Cantwell and Hartzell [1919] made some very interesting statements regarding the effect of lowering the surface tension of media on the growth and habit of bacteria. The protocols of their experiments are unfortunately lacking in details of the methods used for determining surface tension. On lowering the surface tension of their media they observed that anaerobic bacteria grew in the presence of air, that organisms, usually forming pellicles, grew throughout the liquid and did not form a film and that spore-bearing organisms no longer produced spores. Evidently the unusual environment of the bacteria completely changed their habit of growth.

As my interest was in the behaviour of organisms whose pellicle forming properties are important, I devoted some attention to a study of the effects of lowered surface tension on the growth of *C. diphtheriae* and extended these observations to other organisms.

Certain general observations may be made here regarding surface tension, although the subject is discussed among others by Freundlich [1922] and Bayliss [1923].

In the first place no standard method has as yet been arrived at for the determination of its value. In most instances in biological research the stalagmometer has been used and has proved to be a satisfactory instrument if employed in a strictly comparative way. It is in the comparison of the results of one worker with those of another that difficulties arise. This was seen in the meiotagmin reaction of Ascoli and Izar [1910], which is possibly of real biological significance and yet has been found so difficult to repeat by other workers that it has not had general acceptance.

The stalagmometer represents the transition from a purely dynamic method such as the jet method of Lord Rayleigh and the static method of either the capillary tube or the measurement of pull of a solid being torn from the surface of a liquid. Both static and dynamic methods give practically concordant results when pure non-associating liquids are dealt with. It is only when we come to deal with liquids containing substances depressing or elevating surface tension that we find difficulties.

With the drop weight method, the longer the time taken to form the drop, the lower or higher the surface tension value obtained, until a number is reached which is close to that obtained by the static method. That this is so will be seen in some of the present results. The earlier values for bacteriological media obtained with the stalagmometer and only moderately slow dropping are very close to those given by Larson and his co-workers. When the number of drops was decreased and the time taken for each individual drop to fall very much prolonged, values were obtained which were about 10 dynes per cm. lower than in the first method, and these were very close to those obtained by the static ring method to be described later.

Larson, Cantwell and Hartzell [1919] discuss some of the possible causes of the change in the behaviour of bacteria through lowering the surface tension. They point out that the conditions for bacterial nutrition are very different at the surface from those in the interior owing to the great increase in the concentration of nutrient substances such as peptone in the surface layer. This must be correct, for Metcalf [1905] in investigating the behaviour of thin films of peptone solution showed that these consisted of a layer of almost undiluted peptone. We also know that other thin films of protein solutions such as the froth of egg-albumin consist almost entirely of the solid component.

The conclusions of Larson cannot altogether be accepted without reserve. The bacteria/water interface must also present an enrichment atmosphere and before it can be assumed that the air/water interface is a situation of

increased concentration of nutritive substances for the cells we ought to be informed regarding the system chitin/water or hemicellulose/water concentration differentiated with the air/water system before we can generalise respecting situation of increased or decreased nutritional environment.

For lack of more precise information the terms chitin and hemicellulose are here used to designate the composition of the bacterial envelope. It seems more than probable that the composition varies, and it may be this very reason which determines that some bacteria in their search for the most suitable surroundings seek the surface of the liquid while others are attracted to the interior of the medium.

There is also an important effect which is not mentioned by Larson and his colleagues. It is known that in the contest for the surface layer those substances which are more powerful in decreasing the surface tension tend to remove those which are less efficient. This may be the double cause of the deleterious action of small quantities of fats when these are not removed from the medium. They not only lower the surface tension but in so doing expel the nutrient substances of less surface tension-depressing power from the surface of the liquid. Thus the surface layer is no longer as rich in nutrient substances but has a high concentration of substances such as soaps which may be directly toxic to those bacteria existing near the interface. In speaking of the interface not only is the air/water junction implied, but also the bacterial/aqueous interface.

EXPERIMENTAL.

The present experiments were designed to enlarge the observations of Larson, Cantwell and Hartzell, and if possible throw some light on the relation between changes in surface tension and the pellicle formation of *C. diphtheriae*. As Larson and his colleagues had recommended the use of sodium ricinoleate for changing the surface tension of the medium it was decided to employ it. The sample used was made from castor oil by following their directions. A 5 % solution of the soap in water was used as a stock solution.

In the first experiments a stalagmometer of the Traube [1887] type was used, dropping at the rate of 15 drops per minute. The drops fell directly into free air and were counted. As a preliminary, certain combinations of nutrient broth made from English and imported beef combined with 2.0 % of Parke Davis, Witte and Morson's peptones were examined; referring the surface tension to dynes per cm. the following results were obtained:

Table I.

Beef	Peptone	Surface tension in dynes per cm.
Imported	Witte	53.4
"	Parke Davis	58.2
"	Morson	59.6
English	Witte	53.3
"	Parke Davis	58.0
"	Morson	59.6

These results agree fairly closely with those of Larson, Cantwell and Hartzell who found the surface tension of their media to be between 58 and 60 dynes per cm. The principal factor determining the surface tension of the medium appears to be the peptones, and of those used in the present experiments Witte's appears to have the greatest effect. In order to test this, 1.0 % solutions of the three peptones were made up, the specific gravity and surface tension were determined and the effect of equalising the hydrogen ion concentration ascertained. The results were as follows:

Peptone	S.G.	Surface tension dynes per cm.	p_{H}	Surface tension dynes per cm. at p_{H} 7.6
Witte	1.006	54.6	6.6	54.2
Parke Davis	1.005	61.5	6.3	62.1
Morson	1.006	60.5	5.6	60.6

It will be seen that the change produced by reducing the hydrogen ion concentration of the peptone solutions to a common number was slight.

In order to examine the effect of sodium ricinoleate on a nutrient broth, the 5.0 % solution of this salt was progressively diluted and the surface tension of the dilutions determined. They were as follows:

Table II.

Dilution	Sodium ricinoleate %	Surface tension dynes per cm.
0	5.0	32.8
4/10	2.0	33.6
1/10	0.5	39.7
1/25	0.2	47.0
1/50	0.1	52.0
1/100	0.05	57.0

As the surface tension of the peptone broth was already between 55 and 60 dynes per cm., it was decided to use much higher dilutions of sodium ricinoleate. The concentration of the soap in the medium is given in the following table and the surface tension before and after sterilisation:

Table III.

Na ricinoleate %	Surface tension dynes per cm.	
	Before sterilisation	After sterilisation
0.125	40.6	41.2
0.062	43.0	43.4
0.031	48.1	48.4
0.015	51.5	51.6
0.0	59.5	59.2

It will be observed that any hydrolysis of the soap which may have occurred as the result of heating to a high temperature did not measurably affect the surface tension of the media.

The media given in the above table were taken in lots of 10 cc., sterilised

and inoculated with the following rapidly growing organisms: *B. coli*, *C. diphtheriae*, *B. pyocyaneus*, *B. proteus*, *Vibrio septique*, *B. welchii* and *B. subtilis*, and examined after 48 hours' incubation at 37°.

Table IV.

Organism	Concentration of Na ricinoleate %				
	0	0.015	0.031	0.062	0.125
<i>B. coli</i>	+	+	+	+	+
<i>C. diphtheriae</i>	x x +	++	+	?	?
<i>B. pyocyaneus</i>	x x x +	x x x +	x x x +	x x +	x x +
<i>B. proteus</i>	x x x +	x x +	x x +	x x +	x x +
<i>V. septique</i>	x x +	+	?	?	?
<i>B. welchii</i>	+	+	?	?	?
<i>B. subtilis</i>	+	+	?	?	?

Note. x represents pellicle formation; + represents growth.

As will be seen, *B. coli* appears to be relatively indifferent to a concentration of soap which is definitely inhibitory to the anaerobes. Among the more affected is *C. diphtheriae*. This is in agreement with the statements arrived at empirically regarding the necessity of removing all fats from the medium if the growth of this organism is to be satisfactory.

By incubating the cultures for a further 24 hours the differences were accentuated.

Table V.

Organism	Concentration of Na ricinoleate %				
	0	0.015	0.031	0.062	0.125
<i>B. coli</i>	++	++	++	++	++
<i>C. diphtheriae</i>	x x x +	x x x x +	++	+	?
<i>B. pyocyaneus</i>	x x x x +	x x x x +	x x x +	x x x +	x x x +
<i>B. proteus</i>	x x +	x x x +	x x x +	x x +	x x +
<i>V. septique</i>	x +	x x x +	+	+	x +
<i>B. welchii</i>	x +	x x +	+	?	?
<i>B. subtilis</i>	x x x +	x x x x +	+	+	?

The only additional point of interest in this table is the apparently slight stimulation to growth seen with the lowest concentration of soap used.

This experiment was repeated except that *B. typhosus* was added to the series. *B. sporogenes* showed itself extremely sensitive to the action of soap. *B. typhosus* grew, but with lessened vigour, even in the more concentrated tubes of soap broth.

The next set of experiments was made to test the effect of soaps on glucose fermentation.

For this purpose peptone broth containing varying quantities of sodium ricinoleate was tubed in portions of 10 cc. and sterilised. Each tube contained a Durham tube. To each tube after autoclaving was added 0.2 cc. of a sterile 10 % glucose solution with a sterile pipette. The tubes were placed in the incubator for 24 hours to control their sterility. The tubes were each inoculated with a loopful of actively growing culture and placed in the incubator at 37°.

The results at the end of 24 hours are given in the following tables:

Table VI.

Concentration of sodium ricinoleate %					
	0	0.035	0.052	0.104	0.156
Time—24 hours.					
<i>B. paratyphosus</i> B	++0ggg	+ +0g	+ +0gg	+ +0gg	+ +0ggg
<i>B. subtilis</i>	++ +pp0	000	000	000	000
<i>B. welchii</i>	+ +0ggg	000	000	000	000
<i>C. diphtheriae</i>	000	000	000	000	000
<i>B. murisepticus</i>	++ +pp0	000	000	000	000
<i>B. paratyphosus</i> A	++ +0ggg	+ +0gg	+ +0gg	+ +0g	+ +0gg
<i>B. sporogenes</i>	++ +0gg	000	000	000	000
<i>B. coli</i>	++ +p(?)g	+ +p(?)g	+ +p(?)g	+ +p(?)g	+ +p(?)g
<i>B. pyocyaneus</i>	++ +pp0	+ +pp0	+ +pp0	+ +pp0	+ +pp0
<i>B. typhosus</i>	++ +00	+ +00	+ +00	+ +00	+ +00
<i>B. proteus</i>	++ +0gg	+ +0g	+ +0gg	+ +0g	+ +0g
Time—48 hours.					
<i>B. paratyphosus</i> B	++ +pggg	+ +0g	+ +0gg	+ +0gg	+ +0gg
<i>B. subtilis</i>	++ +pp0	+p0	+00	000	000
<i>B. welchii</i>	++ +0ggg	000	+0g	000	000
<i>C. diphtheriae</i>	+p0	000	000	000	000
<i>B. murisepticus</i>	++ +p0	+ +p0	000	000	000
<i>B. paratyphosus</i> A	++ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
<i>B. sporogenes</i>	++ +0g	+ +0g	+0g	+0g	000
<i>B. coli</i>	++ +0gg	+ +pgg	+ +0gg	+ +0gg	+ +0gg
<i>B. pyocyaneus</i>	++ +p0	+ +p0	+ +p0	+ +p0	+ +p0
<i>B. typhosus</i>	++ +00	+ +00	+ +00	+ +00	+ +00
<i>B. proteus</i>	++ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
Time—96 hours.					
<i>B. paratyphosus</i> B	++ +0gg	+ +0g	+ +0gg	+ +0gg	+ +0gg
<i>B. subtilis</i>	++ +pp0	++ +pp0	++ +pp0	000	000
<i>B. welchii</i>	+ +0ggg	000	+0g	000	+00
<i>C. diphtheriae</i>	++ +pp0	000	000	000	000
<i>B. murisepticus</i>	++ +pp0	+ +pp0	+00	000	000
<i>B. paratyphosus</i> A	++ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
<i>B. sporogenes</i>	++ +0gg	+ +0gg	+ +0gg	+ +0gg	+00
<i>B. coli</i>	++ +0gg	++ +ppgg	++ +ppgg	++ +0gg	++ +pgg
<i>B. pyocyaneus</i>	++ +pp0	++ +pp0	++ +pp0	++ +pp0	++ +pp0
<i>B. typhosus</i>	++ +00	++ +00	++ +00	++ +00	++ +00
<i>B. proteus</i>	++ +0gg	+ +0g	+ +0gg	+ +0gg	+ +0g

Note. The signs used are + = growth, p = pellicle, g = gas. Where 0 occurs the respective characteristic is absent.

It will be noted that among those bacteria which are most susceptible to the action of the ricinoleate are *B. welchii* and *B. subtilis*. Organisms flourishing in the intestine do not seem to be at all susceptible even to large quantities of soap, confirming similar observations of Larson [1922].

In view of the susceptibility of some of these organisms to quite small quantities of soap it was decided to repeat the above experiment, using smaller concentrations, the amount of glucose employed being the same. Only the 24 hours' and 96 hours' results are recorded here.

Table VII.

Concentration of sodium ricinoleate %					
	0	0.012	0.025	0.050	0.075
Time—24 hours.					
<i>B. paratyphosus</i> B	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. subtilis</i>	× × pp0	× × pp0	× × pp0	× × pp0	000
<i>C. diphtheriae</i>	× × pp0	000	000	000	000
<i>B. murisepticus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. paratyphosus</i> A	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0g
<i>B. coli</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. pyocyaneus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. typhosus</i>	× × 00	× × 00	× × 00	× × 00	× × 00
<i>B. proteus</i>	× × 0gg	× × 0g	× × 0g	× × 0gg	× × 0g
<i>B. enteritidis</i>	× × pp0g	× × 0gg	× × 0g	× × 0g	× × 0gg
Time—96 hours.					
<i>B. paratyphosus</i> B	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. subtilis</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>C. diphtheriae</i>	× × pp0	× pp0	000	000	000
<i>B. murisepticus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. paratyphosus</i> A	× × 0gg	× × 0gg	× × 0gg	× × 0gg	× × 0gg
<i>B. coli</i>	× × 0gg	× × 0gg	× × 0gg	× × 0g	× × 0g
<i>B. pyocyaneus</i>	× × pp0	× × pp0	× × pp0	× × pp0	× × pp0
<i>B. typhosus</i>	× × 00	× × 00	× × 00	× × 00	× × 00
<i>B. proteus</i>	× × 0gg	× × 0g	× × 0g	× × 0g	× × 0g
<i>B. enteritidis</i>	× × 0gg	× × 0gg	× × 0gg	× × 0g	× × 0g

It will be noted that here again *C. diphtheriae* shows itself unusually susceptible to small concentrations of soaps.

The next series of experiments was directed towards ascertaining whether changes in the surface tension of the medium took place during growth of *C. diphtheriae*. The reason for this was partly the interesting statements of Warden and his co-workers [1921] on the constitution of toxins in general. If toxin production be contingent on the dispersion of fatty acids on certain colloid aggregates, then one would anticipate that changes would occur in the surface tension of the medium during the transfer. Furthermore, it might be expected that during the early growth of the bacteria and the consequent introduction of large colloidal particles, the surface tension-lowering substances already present in the medium would be adsorbed by the bacteria with a rise in their surface tension.

In order to get better results than had been obtained with the original form of Traube stalagmometer it was decided to use a drop weight method with a much slower rate of drop and to weigh the drops as formed. In the first set of experiments 30 drops were weighed and these were collected in a small weighing bottle which enclosed the tip of the stalagmometer. In this way disturbances from currents of air and from evaporation of the liquid were avoided. The total time for dropping was 3½ minutes. The average weight of a drop of water from this tip was 0.105 g.

The results with this method were very satisfactory from a comparative point of view, quadruplicate weighings of 30 drops of water did not differ by more than 0.2 %. With peptone solutions the results were not so concordant, a difference of 1.0 % being obtained.

A series of very careful experiments was then made in which five drops of the medium were weighed, the time taken for a single drop of water to fall being about 45 seconds. Excellent results were given by the slow method of drop, and as was to be expected the values for surface tension approximated to those given by the static method when peptone solutions were tested. The differences obtained did not appear to justify the employment of the slower method of drop.

A series of tubes of bouillon peptone was set up and inoculated with the pellicle of a rapidly growing culture of *C. diphtheriae*. At stated times a tube of the culture was taken from the incubator, the culture centrifuged and the clear liquid examined. The results were as follows:

Hours	Surface tension dynes per cm.
control	57.8
24	58.2
72	58.9
120	55.8
168	58.2

It will thus be seen that during the early period of growth the surface tension rose owing to the adsorption by the bacteria of surface tension-lowering substances in the original medium. Following this there is a discharge of these materials into the medium, due, probably, to death and autolysis of bacteria, and the surface tension falls. The last determination gives a high value, but whether this is associated with other changes in the medium cannot be stated.

A second experiment was made in which not only the surface tension was determined, but at the same time the reaction and the production of carbonates were estimated. In this experiment quantities of bouillon of 100 cc. were inoculated. These were contained in flat medicine bottles which were incubated lying on their larger sides. The surface of medium exposed to the air was about 115 cm. The results were as follows:

Table VIII.

Hours	pH	CO ₂ from 1 cc. medium by acid	Surface tension dynes per cm.
0	8.0	0.09	60.06
72	8.4	0.27	62.20
120	8.4	0.29	62.00
168	8.5	0.30	61.37
240	8.5	0.34	62.70
336	8.6	0.34	62.99
432	8.5	0.32	62.41
768	8.5	0.30	62.90

The results of this experiment run quite differently from the preceding one. In the former it was noted that the surface tension of the medium rose slowly after inoculation to fall later. In the present series the rise occurs but the surface tension of the medium does not fall. The results in both series are the average of very closely agreeing duplicate determinations. In the second series, the surface exposed and the amount of pellicle formed is much greater

than in the test-tube series. This would tend to give a much larger amount of pellicle and leave a greater effective surface for the adsorption of surface tension-lowering substances.

In order to obviate some of the difficulties of the dynamic method it was decided to devise an apparatus which would give static readings. It will not be necessary to mention the various types which are already described in the literature some of which are known to give a high degree of accuracy when properly used.

It seemed that the pull given by a platinum ring would, on account of the ease of cleaning, be the most convenient for the purpose. This method has quite recently been used by du Nouy [1919] who obtained an increasing pull by utilising a torsion wire as had been done by Searle. The apparatus of du Nouy is a very expensive one and it seemed possible that by utilising the device of a dividing chain and ordinary hand scales one could produce the same effect with rather more gradual increase in weight than is afforded by the torsion wire. The essential features of the apparatus (Fig. 1)¹ are a small hand balance (*A*) with arms 8 cm. in length, turning with a load of 2 mg., and a platinum ring (*G*) 0.75 mm. thick, 3.1 cm. in outside diameter, supported from a small platinum ring by three very thin platinum wires. The ends of the rings were soldered together with silver. Care was taken to have the larger ring perfectly flat and in subsequent handling all tendency to deform it was avoided. This was done by always removing the ring with a stout hooked wire fastened to a handle. When not in use the ring was kept in a wide mouthed bottle containing strong sulphuric acid and potassium dichromate. The ring was washed in tap water before use and gently dried over a flame. The rest of the apparatus was made up of "Meccano" parts.

The arrangement of the apparatus was as follows:

The balance was suspended from a framework (*B*) 42 cm. high and 30 cm. wide at the base. The frame was slightly sprung inwards at the top and suitably braced to increase its rigidity. Holes were drilled in the horn pans (*C* and *D*) and from the right hand pan a small gold coated eyeglass chain (*F*) fell which ended in a hook supporting the platinum ring (*G*).

From the other fell a much longer (*E*) chain which was doubled upwards and was fastened to a hook which could be made to travel up and down by means of a rack and pinion (*H*). The position of the hook was determined by a fixed millimeter scale (*L*). By lowering the hook, an increase in weight, due to increase in the pan portion of the chain, was put upon the pan. By adding weights to the right hand pan (*C*) it was easy to construct a curve giving the increments of weight on the millimeter scale. In the present apparatus one millimeter roughly corresponded to 2 mg.

An improvement to the apparatus as drawn consisted in gearing down the rate of rotation of the wheel by means of a second smaller wheel fixed near

¹ I am indebted to Mr Colbrand of the Engineering School for the isometric scale drawing of the apparatus.

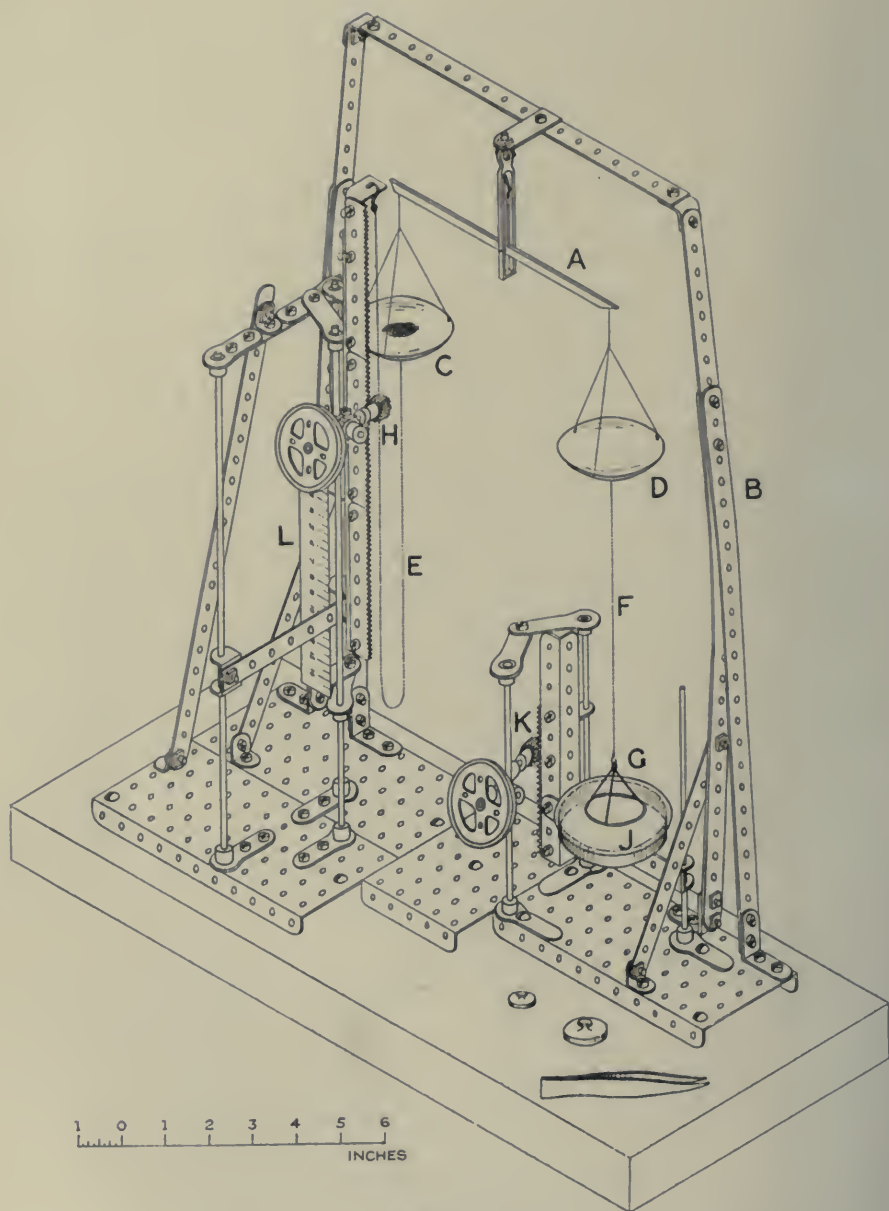


Fig. 1

the bottom of the pillar and connected with the former by a small catgut driving cord. The handle of this spindle was made from the largest sized meccano wheel. This slight alteration is a considerable improvement in the smoothness of movement of the ratchet.

The vessel used for holding the liquid to be examined was a large watch glass (*J*). This was supported on a platform which could also be raised and lowered by a rack and pinion (*K*) so that when the platinum ring was immersed in the fluid, after being balanced without contact, the balance could be returned to the zero point. The movable platform allowed the liquid to be raised gently until contact with the platinum ring was secured.

All determinations were made at room temperature which was 16–18°. For more precise work the container or the whole apparatus could be enclosed in a simple thermostat. Using fresh tap water to determine the water constant of the apparatus the weight required to tear the ring from the surface was 1.698 g.

The method of making a determination was as follows.

The scales were balanced by shot so that the pointer for the millimeter scale was at zero. The watch glass was provided with a few cc. of the solution to be examined, and was slowly moved up until the platinum ring was just in contact with the surface of the fluid. Weights were then added until the ring was being pulled away from the liquid without breaking contact. The weights were gently placed on the scale pan with as little disturbance as possible. With a little experience one easily recognises when it is time to have recourse to the chain for finally increasing the weight. At this point, by means of the rack and pinion on the left, the chain weight is *gradually* lowered. This should be done with as even and slow a motion as possible. At a certain point the ring is torn from the liquid and readings of the weights in the pan and on the millimeter scale are noted, and the scale reading converted into milligrams from the plotted curve. This is added to the pan weights and from the total weight the surface tension of the liquid is deduced. The experiment is then repeated to ensure concordance. With a total weight of over 1 g., individual experiments do not vary much more than 5–7 mg., so that the apparatus acts with an accuracy well under 1%. As was to be expected the values obtained for the surface tension of solutions of peptones were considerably below those obtained by the ordinary drop methods. On an average the values were about 7–10 dynes per cm. lower. This difference was almost entirely due to a function of the dropping rate of the stalagmometer, for the experiments with the drop weight method in which the drops were produced at a very slow rate gave results very close to those obtained with the static method.

An experiment with *C. diphtheriae* will indicate to what extent growth of the organism in a peptone broth medium affects the surface tension. The strain used was P.W. 8 growing actively in the same medium. The culture used for inoculation was two days old. These experiments were performed in test-tubes.

The initial surface tension of the medium was 50.2 dynes per cm.; the p_{H} 7.8.

The following table gives the curve of the surface tension as measured by the static method:

Table IX.

Hours	p_{H}	Surface tension in dynes per cm.
0	7.8	50.2
24	—	52.2
48	6.8	47.1
72	7.5	45.7
120	8.2	47.6
144	8.5	46.9
192	8.5	45.6
240	8.5	45.2
288	8.5	45.6

This table also gives the interesting result that during the very early stage of growth some of the substances which produce a fall in surface tension are apparently adsorbed on the bodies of the young bacteria and therefore the surface tension rises. With death and autolysis, which occur later, fatty substances are liberated and with this the tension falls. It is noteworthy that this is not coincident with an alkaline reaction of the medium, for at the 48th hour, when the nutrient broth had a reaction of p_{H} 6.8, the tension dropped to 47.1 dynes per cm. It thus agrees with the first experiment in which the surface tension was determined by the drop weight method. This experiment was also performed with test-tube cultures.

The differences due to the media being contained in deep layers in test-tubes or in shallow layers with a larger surface exposed to air were so striking, that it was decided to repeat them in shallow layers on a larger scale and to use for comparison strains which were known to be toxigenic and those which were non-toxigenic. There were also drawn into the investigation three media which experience has shown to be the most suitable for the production of toxin.

Two strains of non-toxigenic *C. diphtheriae* were used, which were obtained from the National Type Collection at the Lister Institute. The toxigenic strain was P. W. No. 8. Two cultures of this strain were used, one of which had been subcultured in this laboratory for some years, the other being a fresh culture from the Lister Institute. No difference in behaviour between the two cultures was noticed. The three media employed were (1) the ordinary peptone broth, (2) Hartley's [1922] modification of Douglas's medium and (3) Martin's medium made up in accordance with the direction of Abt and Loiseau [1922].

The media were sterilised in 1300 cc. Fernbach flasks containing 500 cc. of medium. The necks of the flask were plugged with cotton wool. Through the neck passed a glass tube the upper part of which was bent at a right angle. This was provided with a rubber tube and screw clip. By attaching the rubber tube to a small wash bottle and gently sucking, a small amount of the

contents of the Fernbach flask could be removed without disturbing the flask in the incubator. The samples drawn off were usually so clear that it was not necessary to centrifuge them. The device is similar to that employed by Wolf and Harris [1917] in their study of anaerobic fermentation except that the cultures are maintained aerobically.

Only sufficient of the medium was withdrawn to determine the hydrogen ion concentration and the surface tension. At the end of the experiment the culture was examined for contamination. None such was ever found. Only one of the three experiments will be given although the qualitative results were very similar.

Table X.

Hours	Medium											
	Martin				Douglas				Peptone broth			
	P.W. No. 8		Gentry		P.W. No. 8		Gentry		P.W. No. 8		Gentry	
	<i>p_H</i>	S.T.	<i>p_H</i>	S.T.	<i>p_H</i>	S.T.	<i>p_H</i>	S.T.	<i>p_H</i>	S.T.	<i>p_H</i>	S.T.
0	7.5	44.8	7.5	44.9	7.5	46.6	7.5	45.7	7.6	44.9	7.6	45.7
24	7.2	45.6	7.1	48.6	7.0	48.0	7.0	48.1	6.8	47.8	6.6	48.3
48	7.4	47.0	7.2	49.1	7.2	48.0	7.3	49.6	6.6	48.4	6.8	50.3
72	8.0	48.3	7.6	52.8	7.7	49.0	7.8	53.0	7.2	48.6	7.5	54.1
96	8.3	49.2	8.2	54.0	8.0	50.0	8.0	53.1	7.7	49.0	8.0	54.7
120	8.4	50.2	8.4	55.1	8.2	51.6	8.2	52.6	8.0	51.3	8.2	55.4
144	8.4	51.1	8.5	55.4	8.2	52.0	8.4	54.6	8.2	51.6	8.4	57.8
168	8.4	51.6	8.5	54.9	8.2	51.9	8.4	54.1	8.1	51.2	8.5	57.4
264	8.5	52.6	8.6	57.8	8.4	54.0	8.6	55.7	8.4	53.7	8.7	58.6
384	8.5	52.8	8.7	57.5	8.6	52.8	8.6	53.8	8.6	51.6	8.7	58.2
576	8.8	54.8	8.8	55.3	8.7	53.3	8.8	54.3	8.8	51.6	8.8	57.9

The main point to be observed is the continuous rise in the surface tension of the medium during the growth of the culture and what is more important during a time when it may be assumed that growth has practically ceased.

Speaking generally it may be assumed that the toxin production under the conditions of the experiment had reached its maximum at the fifth day. Curves were drawn of the rise in surface tension and it was found that this rise was, on the whole, steepest during the first 120 hours of the experiment. What is more striking is the divergence of the curves for a given medium produced by a toxigenic and a non-toxigenic strain. In each pair of experiments the curve of rise in surface tension given by a non-toxigenic strain is much steeper, and the ultimate rise in surface tension much higher than with a toxigenic bacillus. This would lead one to believe that the adsorption of surface tension-lowering substances by the bodies of the bacteria is more complete when little toxin is formed.

It may be that the envelope of the bacteria, by reason of its content in fat plays an important part in determining toxin production. Those strains of bacteria whose outermost coating has the greater amount of fat will not adsorb the metabolic products of surface tension-lowering capacity on which toxin production depends. That such substances are elaborated is indicated, not only by the experiments of Warden to which allusion has already been made, but also by the more recent investigations of Walbum [1923] and Doyon

[1922]. Walbum, in particular, believes that a non-toxic "protoxin" is elaborated by the bacillus, and this in contact with peptone or peptone broth produces the active toxin. One may take it that the "protoxin" is of a surface active character, and if adsorbed on the bodies of the bacteria would be removed from a sphere where toxin can be formed. This would give a partial explanation for the fact that non-toxic strains of bacteria remove the surface tension-lowering substances from the medium.

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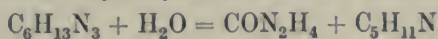
C. THE CONSTITUTION OF GALEGINE.

BY GEORGE BARGER AND FRANK DAVID WHITE.

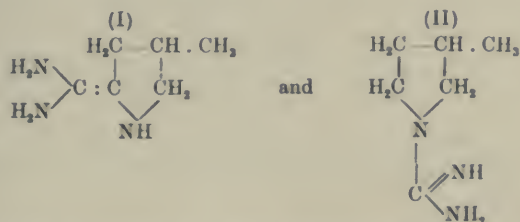
From the Department of Medical Chemistry, University of Edinburgh.

(Received September 19th, 1923.)

TANRET [1914] isolated from the seeds of *Galega officinalis* a base of the formula $C_6H_{13}N_3$, which is hydrolysed by baryta according to the equation:



into urea and a volatile base; the latter Tanret (erroneously) regarded as 3-methylpyrrolidine, which led him to suggest two possible formulae for galegine:



Neither formula accounts for the properties of galegine, as given by Tanret. According to both, galegine would possess an asymmetric carbon atom; yet this base has no optical activity, nor could Tanret resolve it. Moreover, substances of either constitution would give monobenzoyl derivatives (or in the case of formula (I) possibly a tribenzoyl compound); yet Tanret obtained a dibenzoyl derivative of galegine. These discrepancies induced us to re-investigate this question, particularly since the formation of urea by hydrolysis suggested that galegine is a guanidine derivative, and naturally occurring guanidine derivatives are rare. Tanret had indeed already considered its relationship to arginine (see note at the end of this paper).

We soon established the presence of a guanidine nucleus, because galegine gives Weyl's test and the diacetyl reaction [Harden and D. Norris, 1911] and is precipitated by silver nitrate in ammoniacal solution. The correct identification of the other fission product $C_5H_{11}N$ was a more lengthy process. Tanret had found that the boiling point of this base ($105-108^\circ$) and the melting point of its platinichloride ($194-196^\circ$) correspond closely to those recorded for 3-methylpyrrolidine ($103-105^\circ$ and $194-196^\circ$ respectively); the agreement with the corresponding data for piperidine (b.p. 106° , m.p. of platinichloride $198-200^\circ$) is, however, almost as good. But when we had prepared the corresponding aurichloride and picrate, it was evident that the base was not identical with either 3-methylpyrrolidine or with piperidine. In order to choose between

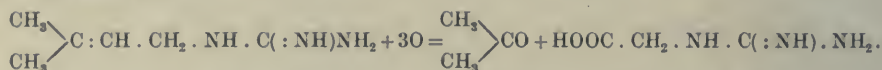
the several other possible constitutions, a closer examination was necessary, which yielded the following results:

1. The volatile base gives Hofmann's carbylamine reaction.
2. Treated according to Hinsberg, with toluenesulphonyl chloride it gives a toluenesulphonamide soluble in sodium hydroxide.
3. It decolorises potassium permanganate in dilute sulphuric acid solution [Willstätter, 1900].

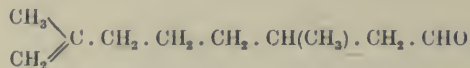
Observations 1 and 2 prove the base to be a primary one, observation 3 proves it to be unsaturated: it is therefore an amino-amylene.

In conformity with this view, galegine was found to take up exactly one molecule of hydrogen when reduced with palladium as catalyst, and the resulting dihydrogalegine yielded on distillation with lime *iso*-amylamine.

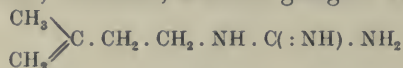
Now only the position of the double bond in galegine remained to be determined. This was done by oxidising galegine sulphate with barium permanganate, which yielded both acetone and glycocyamine (guanidino-acetic acid). The constitution was now almost established, for the oxidation may be expressed thus:



We say "almost established" for in the analogous case of citronellal, it was shown by Harries and Schauwecker [1901], after much controversy, that the constitution is



which does not prevent the formation of acetone [Tiemann and Schmidt, 1897]. It is a fine point, therefore, whether galegine has not the constitution



instead of that given in the above equation. It may perhaps be possible to settle this by a similar method to that employed by Harries and Schauwecker [1901] for citronellal.

Boiled with dilute sulphuric acid, galegine becomes stable to permanganate and takes up a molecule of water, forming hydroxydihydrogalegine; probably the hydroxy group is attached to the tertiary carbon atom.

Although the constitutions of galegine and its hydration product have still slight elements of uncertainty, such uncertainty does not arise in the case of dihydrogalegine; its constitution was, moreover, placed beyond doubt by its synthesis from *iso*-amylamine and cyanamide. The analogous synthesis of galegine itself would require the unsaturated amine $\text{C}_5\text{H}_{11}\text{N}$; very few bases of this type are known, for their synthesis presents considerable difficulty. It is indeed remarkable how few unsaturated simple bases occur in nature. Galegine is even more interesting in this respect than in the possession of a guanidine nucleus.

Methods of isolating galegine from the seeds.

The seeds were obtained and extracted for us by the British Drug Houses, Ltd. for whose help we are much indebted. From a portion of the extract galegine sulphate was first isolated by following Tanret's directions exactly, but we found his elimination of acetic acid by ether and removal of the sugar very tedious and afterwards greatly simplified the process. The extract (obtained after evaporating the 60 % alcohol used in extraction) contained much fat and was ground up with sand to a stiff paste and extracted several times with cold water. The combined decantations and final filtrate were treated successively with normal and basic lead acetate, the lead was removed as sulphate, the excess of sulphate ions quantitatively by baryta, and the filtrate was evaporated to a thin syrup. On adding 50 % sulphuric acid until acid to Congo the galegine sulphate ultimately crystallised in a yield of 0.5 % of the seeds, the same as that obtained by Tanret. After we had found that galegine is precipitated by silver nitrate and ammonia, we worked up the mother liquors by Kossel and Kutscher's method for the isolation of arginine, but only obtained a very small additional amount. Galegine, being unsaturated, is even more readily oxidised by silver in alkaline solution than creatinine [cf. Ewins, 1916]. In any case the solubility of galegine sulphate in cold water is so small, that but little is lost in the mother liquors. We also used an alternative method of isolation by precipitation with potassium bismuth iodide, regenerating with lead hydroxide. The resulting syrup would not at once yield the sulphate; it indeed readily gave the picrate, but as the regeneration of the latter is troublesome (see below) this alternative method is not recommended.

Colour reactions of galegine.

(a) Weyl's reaction. 2 cc. of a very dilute solution of sodium nitroprusside is made alkaline with 2 drops of 10 % sodium hydroxide and one drop of a 1 % solution of galegine sulphate is added; after standing for 15 minutes a red coloration had developed; limit 1 : 6000.

(b) Diacetyl reaction. One drop of diacetyl (prepared according to Diels and Stephan [1907]) is dissolved in 5 cc. of water and 2 drops of 10 % sodium hydroxide are added. On further adding one drop of a 0.5 % solution of galegine sulphate and warming slightly, the characteristic pink coloration of guanidine derivatives is produced. Limit 1 : 10,000. Under similar conditions we found the limit for arginine nitrate 1 : 5000.

Reduction of galegine; dihydrogalegine and its salts.

0.88 g. galegine sulphate (5 millimols of base) was dissolved in 25 cc. of water, 0.02 g. palladium chloride in a little very dilute hydrochloric acid was added, and the solution was shaken in a hydrogen atmosphere under a pressure of 2 atmospheres. The absorption was complete in three hours; no gum arabic was necessary.

Hydrogen absorbed: 115 cc. at 11° and 752 mm. = 109 cc. at N.T.P.

Hydrogen calculated for 5 millimols 112 cc. „

The catalyst, which had coagulated, was filtered off, and the solution was evaporated to a syrup. On dissolving this in boiling alcohol and cooling, dihydrogalegine sulphate crystallised in colourless prisms, M.P. 270° (galegine sulphate melts at 227°).

0.2033 gave 0.1336 BaSO₄ = 27.6 % H₂SO₄; (C₆H₁₅N₃)₂H₂SO₄ = 27.2 % H₂SO₄.

The sulphate is only slightly soluble in alcohol and water, yet more so than galegine sulphate. Weyl's and the diacetyl reaction are similar to those with galegine, but less sensitive. Permanganate and bromine water are not decolorised.

Dihydrogalegine nitrate is little soluble in dilute nitric acid but fairly readily in water and alcohol, and forms long needles from alcohol and ether, M.P. 75–76°.

Dihydrogalegine picrate is almost insoluble in cold water and crystallises from hot water in long narrow plates, M.P. 172°.

1 g. of the dry sulphate was mixed with 4 g. of quick lime and distilled. Besides ammoniacal vapours, it yielded (1) a colourless liquid, B.P. 89–95°; (2) a small fraction, B.P. 95–105° from which minute crystals separated, resembling cholesterol; (3) a yellow viscous mass, remaining in the flask, partly sublimed and partly distilled at 175°/0 mm., forming an oily liquid which solidified to a partially crystalline wax. This was insoluble in dilute acids, but soluble in concentrated hydrochloric acid, from which it was precipitated by ammonia. It is also soluble in glacial acetic acid and yields a picrate, after the solution has been diluted by water. A similar substance has been observed by Tanret on distilling galegine. We mention its production in the present case as evidence that its formation is independent of the unsaturated linking. The feeble base, which is formed perhaps from any alkylguanidine, requires further investigation.

The first fraction of the distillate was redistilled twice over solid potassium hydroxide and then over a mixture of sodium and potassium when it boiled pretty constantly at 95–96°. It had a strong ammoniacal odour, fumed in air and readily formed a carbonate, gave a white precipitate with Nessler's reagent, was stable to dilute acid permanganate and gave the carbylamine reaction.

The hydrochloride (long thin plates from acetone) M.P. 215° and the picrate M.P. 130–134° were found to be identical with the corresponding salts prepared from commercial *iso*-amylamine. A mixture of the hydrochlorides melted at 214°, of the picrates at 130–133°. *Iso*-amylamine picrate was prepared by adding an ethereal solution of picric acid to the amine, and recrystallised, on standing, from an ethereal solution containing a little acetone. On rapidly evaporating the ethereal solution, this picrate was obtained in fine needles. The amine from dihydrogalegine is therefore *iso*-amylamine.

Synthesis of dihydrogalegine (iso-amylguanidine).

Following the method used by Kossel [1910] for the synthesis of agmatine, we kept a concentrated aqueous solution of 1 g. cyanamide and 1.9 g. *iso*-amylamine for ten days at room temperature. Unchanged *iso*-amylamine was then boiled off and a cold saturated solution of picric acid added. A voluminous precipitate was immediately formed; recrystallised from boiling water, it formed long narrow plates, similar to those of dihydrogalegine picrate; yield 2.1 g. = 27% of the theory. On further recrystallisation the picrate melted at 171° (dihydrogalegine picrate from galegine 172°). A mixture of the two specimens melted at 171–173°. The regeneration of this picrate, like that of galegine, was difficult on account of its very slight solubility and the fact that guanidine derivatives are powerful bases. Boiling with 10 % sulphuric acid only decomposed an insignificant proportion. The method finally adopted was to dissolve in warm glacial acetic acid, dilute with ether, and extract many times with dilute sulphuric acid. After washing the aqueous solution with ether and removing the sulphate ions quantitatively with baryta, a syrup resulted on evaporation, which on addition of nitric acid yielded the nitrate. This after recrystallisation melted at 75–76°, and, mixed with the nitrate of the reduction product of galegine, at 74–76°.

This further proves the identity of dihydrogalegine with *iso*-amylguanidine.

Oxidation of galegine.

Kutscher [1901] obtained guanidinobutyric acid by oxidising arginine with barium permanganate in alkaline solution. His yield was poor for most of the arginine was oxidised to guanidine. Since we wished to oxidise galegine at the double bond, we considered it better to use only a small quantity of permanganate and to work in acid solution.

0.88 g. galegine sulphate (= 5 millimols of base) was dissolved in 60 cc. of 5 % sulphuric acid and 1.88 g. barium permanganate (= 5 millimols) was added. This could at most yield 5 atoms of oxygen. The purple colour at once disappeared but, although the mixture was boiled, manganese dioxide remained undissolved, and only 3 atoms of oxygen were used up. The mixture of barium sulphate and manganese dioxide was filtered whilst hot, and the excess of sulphuric acid removed from the filtrate with barium carbonate. The solution was then concentrated to half its bulk by distillation, and the distillate kept to demonstrate the formation of acetone (see below). The solution was then evaporated to dryness on the water-bath, when a considerable amount of a solid remained which recrystallised from boiling water in acicular plates. On slow cooling these formed characteristic sheaves; they melted at 270–280°. The substance was little soluble in cold water but readily in both acids and alkalis. It gave the diacetyl reaction, but Weyl's reaction only very faintly and Jaffé's reaction not at all.

Micro-Kjeldahl: 6.21 mg. gave 2.25 mg. N = 36.2 %

4.50 mg. „ 1.64 mg. N = 36.4

Calculated for glycoeyamine, $C_5H_7O_2N_3$ N = 35.9

For purposes of direct comparison we prepared some *glycocylamine* by heating chloroacetic acid with 5 molecular proportions of free guanidine in concentrated aqueous solution to 60° for two hours [Ramsay, 1908]. It was found to be identical with the oxidation product of galegine.

The hydrochlorides were prepared by evaporating a solution of the substance in hydrochloric acid, and formed colourless plates. M.P. (from galegine) 190°; synthetic 191°; mixture 189°.

The picrates formed long needles from hot water. M.P. (from galegine) 201–203°; synthetic 202°; mixture 201°.

As a further confirmation, a little of the oxidation product was heated with concentrated hydrochloric acid to 140° for two hours, so as to convert it into the anhydride, glycoamidine; the residue remaining on evaporation gave a deep blood-red colour with picric acid and sodium hydroxide (Jaffé's reaction).

Acetone was identified as the other oxidation product in the distillate obtained after removal of the sulphuric acid (above). It gave Legal's test with sodium nitroprusside and at once yielded a precipitate with *p*-nitrophenylhydrazine in 50 % acetic acid. The hydrazone crystallised from dilute alcohol in fine yellow needles, M.P. 148–149°. The melting point of the *p*-nitrophenylhydrazone of acetone is given as 148–148.5°. The yield was 0.49 g. or 50% of the theory.

The unsaturated amine C₅H₁₁N from galegine.

As this amine was unknown, we had no material for direct comparison, and on account of the small amount available, we have not yet examined it very closely. We found it most convenient to prepare it directly from galegine sulphate by distillation with lime, as described above for dihydrogalegine; distilled over potassium hydroxide the amine boiled at 105–108°, as in Tanret's experiments; distillation over potassium did not, however, give a sharper boiling point, as it did in the case of *iso*-amylamine. This is evidently connected with unsaturation: potassium partially polymerised the product.

The amine is a primary base (carbylamine reaction; toluenesulphonamide soluble in alkali). It is unsaturated, at once decolorising permanganate and bromine water. It has a pungent, piperidine-like odour.

A rough determination of the density on the small amount of approximately pure distillate gave

$$D_{18^{\circ}}^{18^{\circ}} = 0.779.$$

Although this determination is not accurate, it suffices to establish the fact that the amine is unsaturated and not cyclic. The value $D_{18^{\circ}}^{18^{\circ}} = 0.779$ is much (about 0.06) lower than that of cyclic amines of the formula C₅H₁₁N. Thus piperidine has $D_{18^{\circ}}^{18^{\circ}} = 0.8619$ and 3-methylpyrrolidine has $D_{4^{\circ}}^{0^{\circ}} = 0.8654$. On the other hand the density found is slightly higher than that of the corresponding saturated *iso*-amylamine, from which it differs by the same amount as the densities of an amino-hexylene and the corresponding saturated hexy-

amine. From the densities given in Beilstein (4th ed.) we calculate for *iso*-amylamine $D_{18}^{18} = 0.753$, whence increment for a double bond would be 0.026. The density of $\text{CH}_2 : \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{NH}_2$ is given as $D_{15}^{15} = 0.779$, of $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{NH}_2$ as $D_{20}^{20} = 0.753$, whence a slightly smaller increment for the double bond may be deduced, corresponding to the higher position in the homologous series. For the much lower members of the series, allylamine ($D^{18} = 0.769$ approx.) and propylamine ($D^{18} = 0.720$ approx.) the increment is about 0.049.

In order to obtain the hydrochloride, a solution free from excess acid must be evaporated (alkaline to methyl red, but not to phenolphthalein). Excess of hydrochloric acid fixes itself on the double bond. The hydrochloride is somewhat hygroscopic, and very soluble. It was not analysed, but the following salts were prepared:

Platinichloride, from the solution of the amine in excess of hydrochloric acid, by adding aqueous platinic chloride solution. The yellow precipitate which separates at once, is crystallised from hot water; M.P. 194–197°.

41.9 mg. gave 14.13 mg. Pt	Pt = 33.7 %
Calculated for $(\text{C}_5\text{H}_{11}\text{N})_2 \cdot \text{H}_2\text{PtCl}_6$	Pt = 33.6 %

The same salt was obtained from the amine prepared according to Tanret, by hydrolysis of galegine by baryta at 100° and extraction with ether.

Aurichloride, from concentrated aqueous solutions of the hydrochloride and auric chloride. Rhomb-shaped plates from warm water, M.P. 57°. After drying *in vacuo* over sulphuric acid it sintered at 75° and melted at 81°. It was then analysed.

34.28 mg. gave 16.05 mg. Au	Au = 46.8 %
Calculated for $\text{C}_5\text{H}_{11}\text{N} \cdot \text{HAuCl}_4$	Au = 46.35 %

Picrate. Etheral solutions of the amine and of picric acid are mixed. It is moderately soluble in hot water, from which it crystallises in long narrow plates, M.P. 138.5–139.5°.

Action of hydrobromic and hydrochloric acids on the amine.

In an attempt to prepare the hydrobromide, the amine was evaporated to dryness with excess of hydrobromic acid. The residue, after drying over potassium hydroxide, crystallised from acetone, after adding ether, in fine colourless needles. The salt was not hygroscopic, but unstable to air and light and decomposed on rapid heating to 110°. It was therefore dried *in vacuo* at 78° over phosphorus pentoxide, when it melted at 206°.

Micro-Kjeldahl: 27.15 mg. gave 1.59 mg. N	N = 5.86 %
Calculated for $\text{C}_5\text{H}_{12}\text{N} \cdot \text{Br} \cdot \text{HBr}$	N = 5.67 %

Bromine estimation by direct precipitation with AgNO_3

0.1522 gave 0.2287 AgBr	Br = 63.9 %
Calculated for $\text{C}_5\text{H}_{12}\text{N} \cdot \text{Br} \cdot \text{HBr}$ for both bromine atoms	Br = 64.8 %

The salt is evidently the hydrobromide of a bromo-*iso*-amylamine which readily loses two molecules of hydrobromic acid. Whether the original un-

saturated amino-amylene is thereby reformed, or whether a new cyclic base results, has not yet been determined. In any case this does not appear to be methylpyrrolidine, since the bromo-*iso*-amylamine is not stable to permanganate in sulphuric acid solution.

Similarly chloro-*iso*-amylamine is obtainable by evaporating amino-amylene with hydrochloric acid. The hydrochloride of this chloro-amine is hygroscopic. It was converted into the *platinichloride* $(C_5H_{12}NCl)_2 \cdot H_2PtCl_6$. This is much more soluble than the platinichloride of the unsaturated base, and is decomposed on boiling its aqueous solution. From dilute alcohol it formed iridescent six-sided plates, quite different in shape from the corresponding unsaturated salt, but having the same melting point (193–195°).

This salt decomposes at 110° and was therefore dried *in vacuo*.

35.13 mg. gave 10.88 mg. Pt Pt = 31.0 %

23.93 mg. „ 7.5 mg. Pt Pt = 30.9 %

Calculated for $(C_5H_{12}NCl)_2 \cdot H_2PtCl_6$ Pt = 29.9 %

Evidently this salt had already lost some hydrochloric acid, although the platinum content was still much below that of the unsaturated salt (33.6 %).

The *aurichloride* crystallises from warm water in plates, sintering at 90° and decomposing at 99–101°. It is also unstable, but was obtained practically pure by drying over sulphuric acid.

20.7 mg. gave 8.76 mg. Au Au = 42.3 %

Calculated for $C_5H_{12}NCl \cdot HAuCl_4$ Au = 42.7 %

The *picrate* formed by adding picric acid to the hydrochloride is very much more soluble than the unsaturated salt in water, alcohol or acetone. Washed free from picric acid with ether, it melted at the same temperature as the unsaturated picrate (137–138°) but contained chlorine.

Hydroxydihydrogalegine.

When converting a quantity of (the very slightly soluble) galegine picrate into the sulphate, the former was boiled for some time with dilute sulphuric acid, in order to dissolve it. The resulting sulphate was, however, very soluble in water, in contradistinction to galegine sulphate. It crystallised from methyl alcohol in fern-like crystals melting at 205–206°. (Galegine sulphate m.p. 227°; the mixture melted at 200°.)

The new sulphate was quite stable to potassium permanganate and to bromine water; it did not take up hydrogen in the presence of palladium, but it still gave Weyl's and the diacetyl reaction. The air-dry substance contains one molecule of water of crystallisation.

Dried at 120–130° it lost 4.1 %.

$(C_6H_{15}ON_3)_2 \cdot H_2SO_4 \cdot H_2O$ requires $H_2O = 4.4$ %.

The anhydrous substance was analysed.

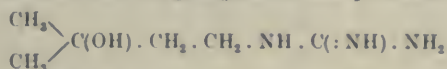
0.2300 gave 0.3091 CO_2 and 0.1696 H_2O C = 36.65; H = 8.2.

8.69 mg. (micro-Kjeldahl) gave 1.89 mg. N N = 21.7.

0.2147 gave 0.1300 $BaSO_4$ $H_2SO_4 = 25.4$.

Calculated for $(C_6H_{15}ON_3)_2 \cdot H_2SO_4$; C = 37.1, H = 8.0, N = 21.6, $H_2SO_4 = 25.3$.

On boiling with dilute sulphuric acid galegine therefore takes up a molecule of water, to form hydroxydihydrogalegine of the probable constitution:



The transformation can be conveniently followed by potassium permanganate. Boiling with 25 % sulphuric acid renders it complete in five minutes; with 5 % sulphuric acid about 45 minutes are required.

Hydroxydihydrogalegine picrate separates from a concentrated hot aqueous solution in rhomb-shaped crystals, M.P. 153–154°, much more soluble than galegine picrate.

In order to regenerate galegine from the picrate it is therefore necessary to avoid boiling with mineral acids. It can be done by dissolving in alcohol, diluting with much ether and extracting with successive small quantities of 50 % sulphuric acid. The aqueous solution is washed with ether and the sulphuric acid is removed quantitatively by baryta; the yield is unsatisfactory.

Hydroxydihydrogalegine yields on hydrolysis a hydroxy-*iso*-amylamine, which can also be obtained by boiling the unsaturated amine with sulphuric acid, but has not been examined further as yet. Its synthesis is being attempted, with a view to that of galegine.

This investigation was rendered possible by a grant to one of us (F.D.W.) from the Department of Scientific and Industrial Research. The cost of material and its extraction was met by a grant of the Moray Research Fund of this University. For both grants we wish to express our gratitude.

[*Note added November 22nd.*] This paper was submitted in MS. to M. Georges Tanret, who declared himself in agreement with our conclusions and sent us a copy of a thesis of the University of Paris, 1917, entitled *Recherches chimiques et physiologiques sur la graine de Galega*, in which (p. 34) he had considered in a footnote the possibility that galegine is a guanidyl derivative of an unsaturated *iso*-amylamine; he considered that on hydrolysis with baryta the amine is isomerised to 3-methylpyrrolidine. As we have shown, this latter supposition is incorrect.

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CI. GALUTEOLIN, A NEW GLUCOSIDE FROM *GALEGA OFFICINALIS*.

BY GEORGE BARGER AND FRANK DAVID WHITE.

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(Received September 19th, 1923.)

DURING the preparation of galegine from the seeds of *Galega officinalis* (see the preceding paper) a yellow colouring matter was detected and identified as a new glucoside of luteolin, for which the name *galuteolin* is suggested. It gave a green coloration with ferric chloride, characteristic of catechol derivatives, and was accordingly precipitated by normal lead acetate in the preliminary purification for the isolation of galegine.

Isolation. The precipitate with normal lead acetate, obtained from the extract of six kilos. of seeds, was air-dried, finely ground and extracted several times with boiling dilute acetic acid, in which it was almost wholly soluble. After removal of the lead by hydrogen sulphide and concentration of the filtrate to a small bulk, a yellow solid separated, in a yield of 2 g. This was insoluble in water, and very slightly soluble in absolute alcohol, but more so in hot dilute alcohol from which it crystallised in aggregates of yellow needles melting with decomposition at 280°. These crystals gave the original olive green coloration with ferric chloride, and a yellow precipitate with normal lead acetate, soluble in hot dilute acetic acid. They reduced Fehling's solution and ammoniacal silver nitrate, and dissolved in alkalis with a deep yellow colour. On boiling a small portion with dilute sulphuric acid, cooling and filtering, the filtrate gave Molisch's reaction, suggesting that the colouring matter was a glucoside.

Galuteolin crystallises with 3 molecules of water, of which $2\frac{1}{2}$ are lost at 120–130°, but the remaining $\frac{1}{2}\text{H}_2\text{O}$ is only removed with great difficulty. This behaviour is similar to that of the glucosides osyretin and violaquercitrin recorded by A. G. Perkin [1902] which both lose $2\frac{1}{2}\text{H}_2\text{O}$ at 130° but the remaining $\frac{1}{2}\text{H}_2\text{O}$ only at 160°. As our glucoside was not stable at the latter temperature, we preferred to dry it at the temperature of boiling tetrachloroethane *in vacuo* over phosphorus pentoxide.

0.1254 air-dry lost 0.0111 at 120–130°;

Calculated for $\text{C}_{21}\text{H}_{20}\text{O}_{11} \cdot 3\text{H}_2\text{O}$

0.1137, dried as above, lost 0.0022 *in vacuo* over P_2O_5 ;

Calculated for $\text{C}_{21}\text{H}_{20}\text{O}_{11} \cdot \frac{1}{2}\text{H}_2\text{O}$

$\text{H}_2\text{O} = 8.85\%$.

$2\frac{1}{2}\text{H}_2\text{O} = 8.96.$

$\text{H}_2\text{O} = 1.93.$

$\frac{1}{2}\text{H}_2\text{O} = 1.97.$

For analysis the glucoside dried at 120–130° was employed.

0.1480 gave 0.2978 CO₂ and 0.0594 H₂O; C = 54.9; H = 4.5%.

0.1538 „ 0.3104 CO₂ „ 0.0635 H₂O; C = 55.0; H = 4.6.

Calculated for C₂₁H₂₀O₁₁ · $\frac{1}{2}$ H₂O C = 55.1; H = 4.6.

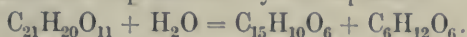
A methoxy determination yielded a negative result.

Hydrolysis of galuteolin. This proceeds readily with quite dilute sulphuric acid [compare A. G. Perkin, 1909].

0.2904 (dried at 120°) was boiled with 20 cc. of 3 % sulphuric acid for 3 hours. The solution was cooled and the precipitate collected and dried; it weighed 0.1808 = 62.3 %.

Calculated for C₂₁H₂₀O₁₁ · $\frac{1}{2}$ H₂O C₁₅H₁₀O₆ = 62.6 %.

The hydrolysis is therefore represented by the equation:



After removal of the sulphuric acid, the filtrate yielded a phenylosazone, which on recrystallisation from alcoholic pyridine melted at 203°. Mixed with a sample of phenylglucosazone it melted at 204°. The sugar is therefore glucose.

The greater part of the available glucoside was now hydrolysed, the resulting insoluble colouring matter crystallising from dilute alcohol in small needles of indefinite melting point, blackening at 290–300°.

After several crystallisations it was dried at 120° and analysed:

0.1305 gave 0.3002 CO₂ and 0.0451 H₂O; C = 62.7; H = 3.8 %.

0.1306 „ 0.3008 CO₂ „ 0.0463 H₂O; C = 62.8; H = 3.9.

Calculated for C₁₅H₁₀O₆ C = 62.9; H = 3.5.

The fission product is readily and completely acetylated by boiling for a minute with acetic anhydride and a drop of pyridine or trace of concentrated sulphuric acid. The acetyl compound, crystallised from absolute alcohol, forms long colourless needles, m.p. 221–223°.

0.1400 gave 0.3104 CO₂ and 0.0496 H₂O; C = 60.4; H = 3.9 %.

Calculated for C₁₅H₆O₆(C₂H₃O)₄ C = 60.8; H = 4.0.

The composition and melting point of the acetyl derivative suggested that the fission product was *luteolin*, and this identity was confirmed by comparison with a specimen of luteolin, for which we are indebted to the kindness of Prof. A. G. Perkin, F.R.S. A portion of the latter specimen was acetylated, and the acetyl derivative, from both sources as well as the mixture, melted at 221–223°.

Sublimation of hydroxyflavones. Before the definite identification of the fission product of our glucoside with luteolin we had already compared it with other hydroxyflavones by a method which does not seem to have been previously employed in this group. We found that in a high vacuum most hydroxyflavones may be sublimed without decomposition. We simply heated minute quantities in a test tube in a metal bath, evacuating with a rotary oil pump (pressure < 1 mm.). The temperature (of the bath) at which sub-

limation takes place, as well as the colour shade of the sublimate, is characteristic, for instance:

	Chrysin	Luteolin	Apigenin	Fisetin
Sublimes at	220°	250-270°	230-290°	230-330°
Resublimes at	220	230-235	260-270	280-290
Colour of sublimate	Almost white	Faint yellow white	Yellowish white	Brownish yellow

Since the melting points of hydroxyflavones are often very high their "mixed" melting points are unsatisfactory. Comparative sublimation (two tubes in the same bath) can be readily carried out with minute quantities.

We found that when galuteolin was similarly heated *in vacuo* a sublimate of luteolin appeared on the sides of the tube at the same temperature as before, and a tarry residue of decomposed sugar remained on the bottom.

In addition to galuteolin, luteolin itself was present in the extract of the seeds. Luteolin has been found previously in a number of plants (*Reseda luteola*, *Genista tinctoria*, *Digitalis purpurea*) but no glucoside of luteolin seems to have been isolated hitherto.

This research was made possible by a grant to one of us (F.D.W.) from the Department of Scientific and Industrial Research, for which grateful acknowledgment is made.

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CII. IMPROVEMENTS IN COLORIMETRY.

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(Received September 20th, 1923.)

SOME years ago I pointed out [Stanford, 1913] that it is not possible to compare the depth of colour of two solutions unless they are of the same shade of colour, and that therefore the colorimetric comparison must be made when the liquids are of the same concentration, and this condition necessarily involves their being of the same thickness of layer. Following this principle, I described in that paper a "dilution" colorimeter, in which the two liquids are contained in similar glass cells, the stronger being then diluted until equality is reached. Whilst that instrument cannot well be criticised from a theoretical point of view, in actual use it is most inconvenient. It is the object of this note to describe improvements which make the dilution colorimeter as rapid and simple to manipulate as any other. These improvements partly concern the colorimeter itself, and partly the source of light employed.

Source of light for colorimetric purposes. The only satisfactory natural source of light for colorimetric purposes is the diffused daylight of a northern sky, but even if this be available its varying intensity is a drawback, because, for any coloured solution, there is a particular intensity of coloration at which a colorimetric comparison is most exact. An unvarying artificial source is therefore preferable.

There is now obtainable under the name of the Sheringham Daylight Lamp a source of light which is identical in quality with the diffused daylight of a northern sky, and does not vary in intensity. Such operations as comparisons in Nessler cylinders can be done with greater ease by this light than by genuine daylight for this reason, and it forms an admirable illuminator for any colour matching instrument.

In this lamp the light from a metallic filament lamp is reflected on to an inverted bowl which is pigmented over strictly proportioned areas with three different pigments, of which a blue predominates. The resulting illumination on a white paper a few inches away is sensibly uniform, and as there is no direct illumination the trouble of actual images does not arise.

Improved dilution colorimeter. The dilution colorimeter in its original form [Stanford, 1913] has the usual pair of rhomboidal prisms, each of which illuminates one-half of the field of view in the eye-piece. In front of the prisms there is a box containing two parallel sided glass cells, one of which

contains the standard solution, and the other the unknown solution. Over each cell is a burette, so that solvent can be run into the stronger solution until equality of colour is reached. The practical disadvantage of the instrument is the eye strain and waste of time involved in constantly withdrawing the eye from the eye-piece and the hand from the stirrer for the purpose of adding cautiously a drop or two more of solvent. The attachment to be described removes this inconvenience. It is merely necessary to pull a string, and drops of solvent issue from the burette as long as the string is pulled and at a rate previously determined by the observer.

Description. The colorimeter as shown in the accompanying drawings (Figs. 1 and 2, which are to scale) consists of two parts, namely, the light box, *ABD*, and the colorimeter proper.

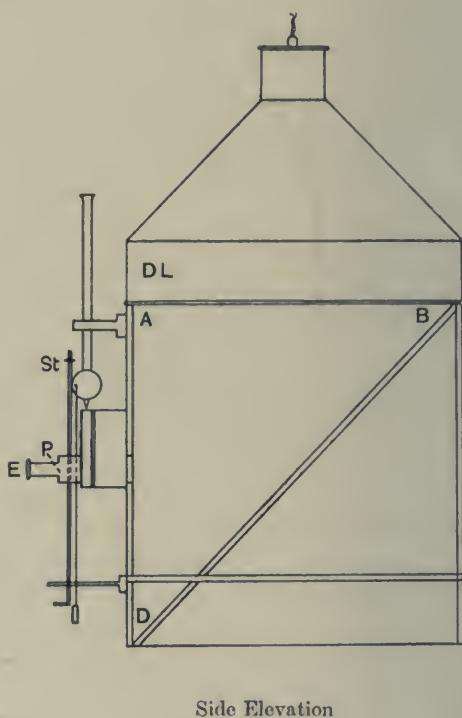
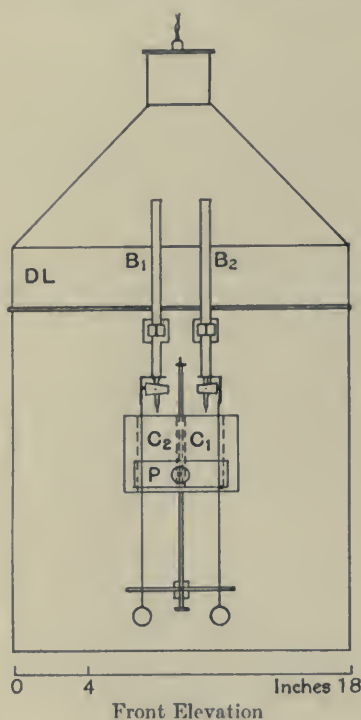


Fig. 1.

The light box consists of a cubical wooden box, each side of which measures 18", bisected diagonally by another piece of three-ply, shown as *BD* in the side-elevation. On the top of the box is a Sheringham Daylight Lamp, *DL*. The particular lamp required is known as type 830*A*, and has a conical reflector 18" in diameter.

The semi-cubical space, *ABD*, is lined throughout internally with white drawing paper, and in the front wall, *AD*, has a horizontal slot centrally situated, and measuring 5" by 1½".

The colorimeter proper consists of a brass box to hold the two cells C_1 and C_2 . This box is screwed to the front of the light box opposite the slot. To the front of this cell-chamber is affixed the case P containing a pair of rhomboidal prisms and the eye-piece E . The stirrup-shaped stirrer St (shown with the stirrup removed) passes through the centre of the prism case. It is spring-loaded, and is worked up and down by means of the finger.

Above each cell is a burette, held in a clip so that it can be easily swung to one side to facilitate removal of the glass cell. The special feature of these burettes is the automatic attachment to the tap which enables them to deliver drops of solvent into the cells at any pre-determined rate. This attachment is shown in side and front elevation in the drawing (Fig. 2). A metal plate C is fixed to the stem of the burette below the tap. This acts as a stop. The burette-tap is firmly gripped by two pieces of wood which are centrally attached to a metal disc (the lid of a small tin is convenient) about $2\frac{1}{2}$ " in diameter. Near its edge this disc carries the studs a , d and e , which are panel-pins held in position with spots of solder, and also the set-pin b . The stud d is attached by means of a spring or rubber band to any convenient anchorage. The stud a carries a thread terminating in a ring large enough to admit the finger. The device acts as follows. Normally, the tension of the spring attached to d brings the stud e against the burette, and in this position the tap is closed.

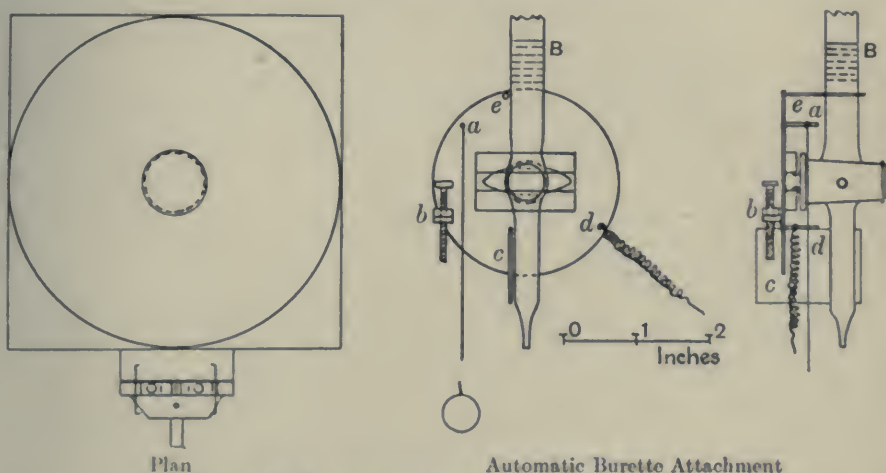


Fig. 2. Improved Dilution Colorimeter.

If now the thread attached to a be pulled down, the tap will open until the end of the set-pin b impinges upon the stop-plate c . By suitably adjusting the set-pin, the rate of drops from the end of the burette can be arranged beforehand. A rate of about one in some three seconds will be found convenient.

In using the instrument all that is necessary, therefore, is to place the two cells containing the liquids in position, switch on the daylight lamp and, while continuously working the stirrer with a finger of one hand, to pull the

string of the burette over the stronger solution, and allow the dilution to proceed until equality of colour is reached. The difference in the readings of the burette gives the volume of solvent added to the stronger solution, and the necessary simple calculation may be made with the help of the formula given below.

Calculation. Let

Total volume of unknown solution be V_u .

Amount of substance contained therein be x .

Volume of solution placed in colorimeter cell be v_u .

Total volume of standard solution be V_s .

Amount of substance contained therein be y .

Volume of solution placed in colorimeter cell be v_s .

Volume of water added in dilution be W .

Then

A. *When standard is diluted:*

$$x = \frac{V_u \times v_s \times y}{V_s \times (v_u + W)}.$$

B. *When unknown is diluted:*

$$x = \frac{V_u \times (v_u + W) \times y}{v_u \times V_s}.$$

Degree of accuracy of the instrument. Some months' use of the instrument has shown that the time required for a colorimetric comparison is about three minutes for any person of average perception in regard to colour.

Apart from this, under the heading of degree of accuracy, the same two points must be dealt with as in all other colorimetric devices, namely, what sources of error may be inherent in the instrument (whether from its principles or from its construction) and what is the degree of accuracy of the actual adjustment of the instrument when equality is reached.

As regards errors due to principles, this colorimeter has none. When equality is reached the liquids under comparison are of the same concentration, they are being viewed through the same thicknesses of layer, and therefore, like Euclid's triangles, they must be equal in all respects.

Under errors of construction come (1) unequal illumination, (2) inequality of the cells, and (3) lack of symmetry of the optical arrangements. Now as to these, the daylight lamp arrangement described entirely obviates unevenness of illumination, which is a fruitful source of trouble in colorimetry as a rule. The internal thicknesses of the cells are tested beforehand by dusting them with lycopodium powder and focussing a measuring microscope first on one surface and then on the other. The two cells should not differ by more than 0.1 %. The symmetry of the optical arrangements is controlled by examining in the colorimeter a number of liquids of different colours, a portion of each being put into each cell. In this connection it may be remarked that the only trouble likely to be met with is an unsymmetrical mounting, or a subsequent displacement, of the rhomboidal prisms.

As a result of a considerable number of experiments, it may be stated that a person with an average eye may expect to get results correct to 1 %, provided the comparisons are undertaken at the most sensitive depth of colour for the particular liquids under examination. [Compare Horn, 1906; Horn and Blake, 1906.] This refers to rapid and solitary estimations. If circumstances permit, say, three independent comparisons to be made, the error of the mean will be considerably less than this.

Mechanical stirrer. The simple form of hand stirrer described in connection with the colorimeter answers very well when only a few estimations have to be made, but becomes tedious and fatiguing if the instrument is in constant use. For this reason a mechanical stirrer is an advantage. It is clear that such a stirrer must be instantly detachable, for both it and the cell in which it works have to be cleaned and dried after each estimation.

Our stirrer is made from a dentist's drilling engine, which consists of a flexible shaft, passing through a flexible cable and carrying at one end a pulley and at the other end a spring bayonet arrangement by means of which the shaft can be instantly connected with the so-called "hand piece." This hand piece contains a small chuck for holding the drills. The actual stirrer is a piece of $\frac{3}{16}$ " glass rod flattened and twisted at the lower end to form a kind of screw, and mounted at its upper end in a socket soldered to an ordinary dental drill. The hand piece is rigidly fixed on a T-shaped piece of wood which slides in two wooden grooves screwed to the front of the light box. When the cells are in place, the piece of wood carrying the handpiece and stirrer is dropped into its slots, the end of the cable pushed on to the bayonet joint, and everything is ready for the estimation. The stirrer is driven by any convenient small motor.

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CIII. NESSLERISATION, AND THE AVOIDANCE OF TURBIDITY IN NESSLERISED SOLUTIONS.

By ROBERT VINER STANFORD.

From the Chemical Laboratory, Cardiff City Mental Hospital.

(Received September 20th, 1923.)

THE brown coloration which is produced when very dilute solutions of ammonium salts are treated with Nessler solution has been used as a means of detecting and estimating ammonia in water analysis for many years, and more recently has found extensive employment in biochemical investigations, where the quantity of material available so frequently necessitates colorimetric analytical methods.

The quantities of substance which are measured in this kind of work are most conveniently reckoned in hundredths of a milligram. To avoid the constant repetition of this clumsy phrase, I venture to coin the word "centimilligram," conveniently written *cmg.*

In the case of every comparison of coloured substances there is a particular concentration at which the comparison is most easily made. This will depend, of course, on the thickness of the layer of liquid which is to be examined, and it varies with different colours, and to some extent with different individuals. Our comparisons are conducted in the dilution colorimeter [Stanford, 1923, 1] using a 10 mm. layer. In these circumstances the most satisfactory results are obtained when the concentration is in the neighbourhood of 1 *cmg.* of nitrogen per cc.

Nesslerisation differs also, however, from the other common colorimetric processes in that it is peculiarly liable to give rise to solutions which are *turbid*, and so useless for colorimetric comparison. This is well known, but until now its cause does not seem to have been discovered. It is stated in Clowes and Coleman's well-known handbook that the Nessler solution must always be added to the ammoniacal solution, and never in the reverse order, or a turbid solution will result. As will be shown, this statement is not correct. In the past few years Folin has described several rapid methods for the estimation of nitrogen or urea nitrogen in products of animal origin "by direct nesslerisation," that is to say, the reaction product is treated with Nessler solution and compared with a nesslerised standard. Cole [1920] has stated that he was unable to repeat these experiments because of the turbidity of the resulting solutions. The trouble seems also to have occurred to Folin himself, for he says, in a later paper, "if the solution be turbid, centrifugalise

a portion of it." A strange counsel, seeing that the turbidity is due to minute oily drops of the dark brown colouring matter which it is proposed to estimate.

After very numerous repetitions of Folin's procedure I could only confirm Cole's statement, but in further investigating the matter I was able to discover the cause of the occurrence of turbidity, and the means of avoiding it. The question really divides itself into two. On the one hand there have to be considered the processes which occur when a pure solution of ammonium sulphate or ammonium chloride is treated with Nessler solution, and on the other hand what occurs when, say, the product of a urine digestion is treated with Nessler reagent.

I have not been able to discover any conditions in which such impure products may be nesslerised to give a clear solution, and it is for that reason that I devised the method for the removal of ammonia from such liquids which is described in the following paper [Stanford, 1923, 2]. Some points with regard to such solutions will be referred to below.

Method of nesslerisation to obtain clear solutions. A clear solution will always be obtained (1) if the concentration of ammonia is below a certain limit (see below) and (2) if the addition of the Nessler solution be made *drop by drop with constant shaking*.

It does not matter whether the reagent is added to the ammonia or *vice versa* provided the addition be carried out in this way, but if the ammonia solution be added to the reagent, the latter should be diluted if possible beforehand.

It ought to be mentioned that two Nessler solutions are in common use, one of which is about twice as concentrated as the other. All the statements in this paper refer to the more dilute solution (about 22 g. KI per litre), but they hold good also for the stronger solution.

Ammoniacal solutions to be nesslerised should not contain more than 2 or 3 cmg. of nitrogen per cc. Turbidity can be avoided when the solutions are a good deal stronger than this, but it is not desirable to try, for a concentration of 1 cmg. of nitrogen per cc. gives a coloration convenient for colorimetric comparison in a 10 mm. layer.

The following experiments demonstrate the whole matter quite clearly:

(1) A solution containing 1 cmg. N per cc. (as $(\text{NH}_4)_2\text{SO}_4$) nesslerises clear when treated with one-tenth of its volume of Nessler solution added drop by drop with constant shaking.

(2) Repeat No. 1 without shaking and a turbid solution results.

(3) Repeat No. 1. The clear solution when treated with a few drops of 20 % sodium hydroxide becomes turbid at once, and the same thing happens if an excess of Nessler reagent be added.

(4) Repeat No. 1, but add to the ammonium sulphate solution a little urease solution (Folin) first; the nesslerised liquid will be turbid even though the reagent be added in drops and with shaking.

Ageing of nesslerised solutions. It is well known that the colour of a nesslerised solution alters rather rapidly with lapse of time. It was hoped that when the solutions were obtained with the precautions above mentioned this alteration might not occur, but unfortunately this is not the case. It is, therefore, still necessary to nesslerise solutions intended for comparison as nearly simultaneously as possible.

SUMMARY.

(1) Pure solutions of ammonium salts always give clear solutions when the Nessler reagent is added drop by drop with constant shaking.

(2) The ammonia solution should not contain more than 3 cmg. of nitrogen per cc., and the Nessler reagent should be one-tenth of the volume of the solution to be nesslerised.

(3) Excess of alkali or of Nessler reagent makes the clear solution turbid, as also does the addition of traces of urease.

(4) No conditions could be discovered in which clear solutions could be obtained by "direct nesslerisation" as suggested by Folin.

I wish to acknowledge my indebtedness to the Medical Research Council, by whom a part of the cost of this work has been defrayed, and also to Mr A. H. M. Wheatley for his cordial assistance.

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CIV. METHOD FOR THE RAPID AND QUANTITATIVE REMOVAL OF AMMONIA FROM SOLUTIONS, ESPECIALLY APPLICABLE TO THE MICROQUANTITATIVE ESTIMATION OF NITROGEN AND UREA IN PRODUCTS OF LIVING ORIGIN.

By ROBERT VINER STANFORD.

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(Received September 20th, 1923.)

MANY analyses of biochemical importance end in the estimation of ammonia. The Kjeldahl process, in one or other of its countless modifications, is everywhere in use, and of recent years the estimation of urea by means of the Soya bean, or of the urease extracted from it, has become almost as much a matter of course.

All these processes leave the nitrogen in the form of an ammonium salt mixed with many other things. Several methods are in use to bring the ammonia into a pure solution in which it can be estimated. The mixture is generally made alkaline, and distilled, or alcohol is first added, or it may be distilled in steam. Another variation is the "aeration method" (drawing a vigorous current of air through it) as proposed by Folin and adopted by van Slyke.

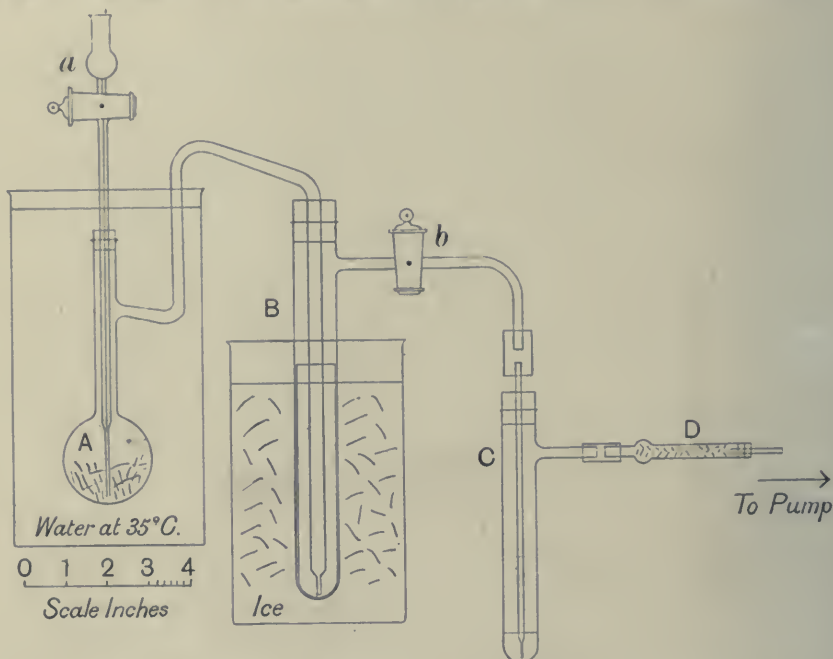
Folin has recently [Folin and Youngburg, 1918] sought a way out of the difficulty by estimating the ammonia by "direct nesslerisation." Unfortunately, owing to the presence of other colloids this nearly always gives turbid solutions (compare preceding paper) which are useless for colorimetric comparison.

The distillation and aeration methods are quite satisfactory as long as the quantity of ammonia to be measured is within volumetric limits, but in biochemical work the quantities to be measured are often so small that volumetric procedure would be absurd. In the case of the total nitrogen, non-protein nitrogen and urea nitrogen of cerebrospinal fluid or of blood, for example, microquantitative methods (in this instance colorimetric) are unavoidable, and it is therefore of importance to have some quick and quantitative means of removing the ammonia from the reaction mixture and getting it in the form of a pure solution which on nesslerisation will give a clear liquid for the colorimeter.

The aeration method is not applicable, for the slight losses which are bound to occur, and which are negligible in, say, a urine analysis, become comparable with the whole quantity of ammonia to be estimated—a quantity which may not exceed a few hundredths of a milligram.

Distillation may be employed, as in my method for the estimation of the total nitrogen of cerebrospinal fluid [Stanford, 1913], but would not be

suitable for urea estimations owing to the decomposition of the urease by the boiling alkali. The distillation method as there described has the advantages that it is easily carried out (except for the actual colorimetric comparison) by unskilled hands and it does not require any special apparatus. On the other hand it consumes a good deal of time, and this is inconvenient if a large number of estimations has to be made. For this reason we now do all our nitrogen estimations with the vacuum apparatus about to be described.



Vacuum Apparatus for the Distillation of Small Quantities of Ammonia.

The apparatus takes the form shown in the accompanying drawing. *A* is a small (100 cc.) distilling flask the side tube of which is lengthened and bent as shown, the end being drawn off to a fine jet. It is connected with the receiver *B* by means of a good rubber cork. In the receiver *B* is placed a thin, rimless test-tube of such diameter as to be an easy fit: this test-tube we term the container. *B* is connected through a tap *b* with the traps *C* and *D* and the pump. The distilling vessel *A* is provided with a capillary which carries a glass tap *a* and above that a small bulb.

The apparatus is used as follows. The "container" is removed from *B*, a wisp of glass wool is put in the bottom of it and the whole is then weighed to the nearest centigram. 4 cc. of very dilute (about $N/20$) sulphuric acid are run in, and the container is then replaced in *B*. The trap *C* is provided with about 3 cc. of dilute sulphuric acid and the tube *D* is filled with glass wool moistened with sulphuric acid. The receiver *B* is immersed in a beaker of ice and water containing enough water to enable the contents of *B* to be observed through it.

The liquid under examination is introduced into the distilling vessel *A*, together with a wisp of glass wool. If the solution is not faintly acid it must be brought to that condition, a scrap of litmus paper being thrown in to act as indicator. It is convenient so to arrange concentrations that the volume of solution taken shall be about 5 cc. The whole apparatus is now connected up as shown in the diagram, the mouth of *B* being previously smeared with glycerol, and, the tap *a* being closed, about 3 cc. of strong (5 *N*) sodium hydroxide solution are placed in the bulb above it. The distilling vessel is now surrounded with a beaker of warm water so that the cork at the top of the flask is completely in the water. The pump is turned on and the apparatus exhausted as completely as possible. With a good water pump this takes less than a minute. The most suitable temperature of the water in the bath will depend of course on the degree of vacuum obtained. A good pump should yield a vacuum of 12–13 mm., and in that case the warm water should be at 35°.

As soon as only an occasional bubble passes through *D*, the tap *b* is closed, and the tap *a* is opened to admit nearly all the sodium hydroxide solution. The apparatus is now left alone for five minutes, care being taken to maintain the temperature of the water-bath by placing a small flame underneath it. A continuous succession of bubbles will be seen being absorbed in *B*. The apparatus is functioning like the old cryophorus. During this period most of the ammonia passes over, and as the distillation is proceeding in a closed space there is no possibility of loss. At the end of five minutes connection with the pump is re-established by gradually opening *b*. Since the cork in *A* is sealed by immersion in water, and the cork in *B* by means of glycerol, no leaks can occur, but the vacuum is generally slightly impaired by reason of dissolved gases in the alkali solution which has been added. When connection with the pump has been completely re-established a fairly vigorous stream of bubbles will be seen passing through *B*, and an occasional bubble through the trap *C*. These are bubbles of water vapour which are only partially condensed in *B*, but they lose there any ammonia they may contain. The distillation is allowed to proceed in this manner for five minutes, counting from the time when the tap *b* first began to be opened. The tap *b* remaining open, the tap *a* is cautiously opened sufficiently to allow the slowest possible stream of air bubbles to pass through the distilling vessel, and this is allowed to continue for five minutes. The tap *b* is then closed, air is admitted through *a* and the apparatus is disconnected generally. The container is removed from *B* and weighed. In this way the total volume of the distillate is known, and it is then nesslerised with 1/10 of its volume of Nessler solution and compared with a standard in the dilution colorimeter.

It is obvious that the whole of the ammonia must be in this distillate unless any has remained in *A* or has passed over into the trap *C*. To check these possibilities, the liquid remaining in *A* is rinsed out into a Nessler cylinder, made up to 50 cc. and nesslerised. If the operation has been properly conducted not more than a barely perceptible coloration will be

observed. The contents of the trap *C* may then be poured in, and should cause no coloration either.

Summary of Procedure. The following summary of the procedure just described in detail should be intelligible on reference to the drawing.

- (1) Exhaust the apparatus.
- (2) Close *b* and admit sodium hydroxide solution through *a*.
- (3) Both *a* and *b* remaining closed, allow the distillation to proceed for five minutes.
- (4) Cautiously re-open *b* and let the distillation proceed a further five minutes.
- (5) Admit the least possible stream of air through *a*, and continue for five minutes.
- (6) Close *b*, admit air generally, re-weigh the container and nesslerise its contents.
- (7) Nesslerise the liquid remaining in *A* and the acid in the trap *C* to check the completeness of the distillation.

Conclusion. Although, like most analytical schemes, rather lengthy in description the process is very quick and simple to carry out, for the distillation takes only 15 minutes, and during most of this time looks after itself.

The transference of the ammonia is almost strictly quantitative, for if any perceptible coloration is shown when the contents of *A* and *C* are nesslerised it does not exceed that due to one or two thousandths of a milligram of ammonia. It is true that even so small an amount makes a regrettable *percentage* error when only a few hundredths of a milligram of substance are there to be estimated, but percentage errors which could not be tolerated in ordinary analytical practice are inseparable from microchemical methods. Consider the experimental errors of the final colorimetric comparison, for instance.

It need hardly be pointed out that a blank experiment must be done to test the freedom from ammonia of any reagents that may have been used in arriving at the solution taken for distillation. Extensive use of the method has been made in this laboratory in connection with estimations of total nitrogen and urea in cerebrospinal fluid and in blood. In the case of the materials we have we find that blank experiments show the presence of less than half a hundredth of a milligram of nitrogen in the quantities of reagents used in any particular analysis, and as this is to a great extent off-set by the traces of ammonia remaining undistilled or passing through to the trap we do not take either error into calculation. Where reagents of a lesser degree of purity are met with the correction must of course be made.

I wish to acknowledge my indebtedness to the Medical Research Council, by whom a part of the cost of this work has been defrayed, and also to Mr A. H. M. Wheatley for his cordial assistance.

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CV. THE AZINE AND AZONIUM COMPOUNDS OF THE PROTEOLYTIC ENZYMES. I.

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(Received September 24th, 1923.)

THE immense importance of the establishment of the chemical nature of the proteolytic enzymes has led to many endeavours to purify them. From the earliest attempts of Schwann [1836] to the present day all these have met with obvious failure.

In 1905 it was observed by T. Brailsford Robertson [1907] that if one drop of saturated safranine (Grübler) is added to a solution of trypsin (Grübler) a precipitate forms which flocculates in the course of a few hours and collects at the bottom of the tube. Some years later Holzberg [1913] demonstrated that the precipitate was proteolytically active. With these two facts in view a study of the nature of this precipitate was undertaken.

Preparation of the Safranine Precipitate.

To prepare a sample of the precipitate, 100 g. of commercial pancreatin (Armour) was extracted with 500 cc. of water and the insoluble fraction filtered off. The amber-coloured solution so obtained was treated with an equal volume of 0.5 % safranine (Grübler) and the resulting precipitate allowed to flocculate for half an hour and then separated by centrifugalisation at high speed for twenty minutes. After washing the resulting fine slimy sediment with 0.5 % safranine, excess of the dye and the water were removed by six successive washings with absolute alcohol. The precipitate was then washed with ether and dried at 40°. The yield constitutes about 2 % of the original pancreatin employed. The precipitate is a red, finely grained powder, which in the presence of water emulsifies to form a violet solution which shows a remarkably strong proteolytic activity when the reaction of the solution is adjusted to p_H 8.0. The compound is hydrolysed by dilute acid (0.2 % HCl) with liberation of the free safranine chloride which may be extracted from its solution by butyl alcohol. The treatment with acid, however, greatly impairs the proteolytic activity owing to inactivation of the dissociated trypsin.

The solution from which the safranine precipitate is removed is completely inactive.

A comparison of the activity of the precipitate with that of the solution from which it was removed, and of the original solution from which it was prepared, is shown by the following experiment.

Comparison of the Relative Digestive Activity towards Caseinogen of the Safranin-Trypsin compound and its Equivalent of Unpurified Trypsin.

Solution A. The trypsin employed was the partially purified and very active product prepared by Gröbler in Leipzig and sold as trypsin puriss. sicc. A 2 % solution in distilled water was used, the small insoluble residue being separated by centrifugalisation. The solution being freshly prepared before each experiment was preserved in ice until wanted.

Solution B. 4 cc. of Solution A were treated with 4 cc. of safranin 0.5 % in a small centrifuge tube with a conical bottom, and after allowing to flocculate for thirty minutes the resulting precipitate was separated in a high-speed centrifuge. The supernatant fluid was decanted and preserved as Solution C. The sediment was washed twice on the centrifuge with 8 cc. of 0.5 % safranin and then six times with absolute alcohol brought to p_H 6.0 with alcoholic HCl. It was then taken up in 4 cc. $M/15$ phosphate buffer (p_H 7.5). 2 cc. of this solution contain the safranin precipitate from 2 cc. of Solution A.

Solution C. This solution is the residue from the precipitation of the azine compound, 4 cc. being equivalent to 2 cc. of Solution A.

The Substrate. Carefully purified caseinogen, prepared by a modification of the method of Van Slyke and Bosworth [1913], was dissolved, with the aid of a mechanical stirrer, in 80×10^{-5} g. equivalents of NaOH (*i.e.* 8 cc. $N/10$ NaOH per g. caseinogen). To this solution, which is neutral to phenolphthalein (p_H less than 8.0), was added 3×10^{-5} g. equiv. of HCl, the addition of the acid being slow and accompanied by rapid stirring. The solution was then diluted so as to contain 4 g. of caseinogen per 100 cc. This solution was diluted with an equal bulk of $M/5$ phosphate buffer solution, prepared by adding 166 cc. $M/5$ Na_2HPO_4 to 34 cc. $M/5$ NaH_2PO_4 , the p_H of which is 7.5. This substrate was preserved by the addition of toluene (0.5 cc.).

Technique of Experiment. 100 cc. portions of the substrate were measured into four long-necked glass-stoppered glass flasks and immersed in a thermostat which was capable of regulating the temperature to within 0.01° . When they had reached the temperature of the bath equivalent portions (2 cc.) of each of the enzyme solutions A, B, C were added to the flasks from which, after shaking, 2 cc. portions were withdrawn by means of a dry pipette and delivered into 2 cc. of $M/6$ CH_3COOH . Subsequently 2 cc. were withdrawn from each at half-hourly intervals and treated similarly. After allowing to flocculate the caseinogen was filtered off and the refractive index of the clear filtrate was determined by means of a Pulfrich refractometer, reading accurately to within 1 minute of the total angle of refraction, the refractive index of the

prism being 1.62098 (experimental error 0.00078 of the refractive index). The amount of caseinogen was determined from the formula:

$$\text{Caseinogen \%} = \frac{2(n-n')}{0.00152},$$

where n is the refractive index of filtrate,

n' is the refractive index of control (prepared at 0 hour's digestion period),

0.00152 is the change of refractive index due to 1 g. of digested caseinogen in 100 cc. of solvent [Robertson, 1912].

The refractive index method of determining the rate of protein hydrolysis has the objection that the experimental error is large, being ± 0.1 g. of the caseinogen in the digest. However, with this difficulty in view, the velocity constant calculated from the mono-molecular reaction was found to be constant within the limits of the experimental error. The results are shown in Table I.

Table I.

Substrate=caseinogen 2 %. $p_{\text{H}}=7.5$. Refractive index = 1.33743. Temperature 40°.

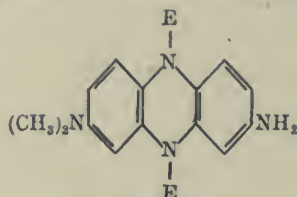
	Time (t) hrs.	Angle of refraction	Refractive index 0 hrs. = n_1	Refractive index at (t) = n	G. digested = $\frac{2(n-n_1)}{0.00152}$	a	x	$K = \frac{1}{t} \log_{10} \frac{a}{a-x}$
Enzyme solution A, 2 cc. (original solution)	0	66° 39'	1.33591	1.33591	0.0	—	—	—
	$\frac{1}{2}$	66 36	1.33591	1.33614	0.3	2	0.3	14×10^{-2}
	1	66 33	1.33591	1.33639	0.5	2	0.5	12
	$1\frac{1}{2}$	66 31	1.33591	1.33655	0.8	2	0.8	14
	2	66 29	1.33591	1.33671	1.0	2	1.0	15
Enzyme solution B (azine pre- cipitate from 2 cc. of A)	0	66 39	1.33591	—	0.0	—	—	—
	$\frac{1}{2}$	66 37	1.33591	1.33606	0.2	2	0.2	9
	1	66 35	1.33591	1.33623	0.4	2	0.4	9
	$1\frac{1}{2}$	66 32	1.33591	1.33647	0.6	2	0.6	10
	2	66 31	1.33591	1.33655	0.7	2	0.7	9
Enzyme solution C (residue from azine precipitate B)	0	66 39	1.33591	1.33591	0.0	—	—	—
	2	66 39	1.33591	1.33591	0.0	—	inactive	—

The table explains itself. The azine precipitate which constitutes only approximately 2 % of the preparation has retained at least 70 % of the activity shown by the original enzyme solution. The loss may be due to partial inactivation of the enzyme during the rather rough treatment it receives, but, in all probability it is due to the incomplete dissociation of the enzyme-azine compound. The solution from which the azine precipitate was removed was completely inactive.

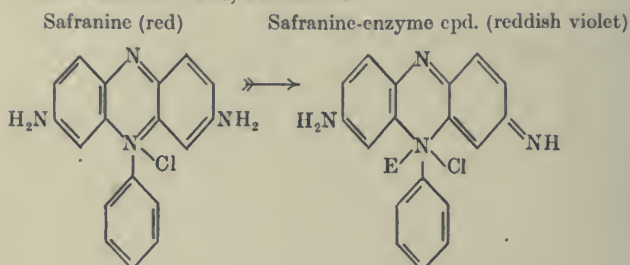
With the possibility in view of determining the nature of the linkage between the enzyme and the safranin molecule, the action of the other azine bases (eurhodines, safranines, indulines) towards the proteolytic enzyme trypsin was determined. Of the series tried, all those compounds which were water-soluble and contained the azine nucleus were capable of completely precipitating the enzyme from its solution.

Thus dimethyldiaminophenazine hydrochloride (neutral violet), dimethyldiaminotoluazine hydrochloride (neutral red), diaminophenyltoluazonium

chloride (safranine), dimethyldiaminophenylphenazonium chloride, all quantitatively precipitate the active principle from an impure trypsin solution. Even the complex aminodianilinophenylphenazonium chloride (indamine blue) is active in the precipitation. The type of union between the enzyme and azine base proved to be a direct combination with the azine nitrogen. In the case of dimethyldiaminotoluazine hydrochloride the union would be represented thus, where E represents the Enzyme molecule:

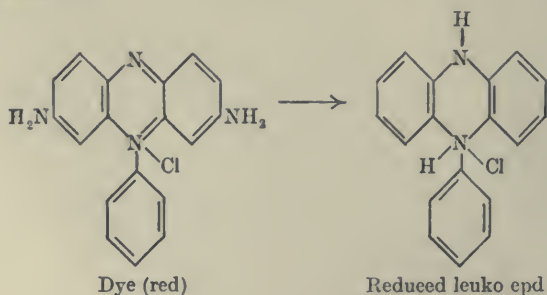


or in the case of the azonium base, safranine



The mono-enzyme compound would alter the constitution from the ortho-para-quinoid structure, with a consequent tautomeric rearrangement and accompanying colour change. The colour change, from red to violet, is noticeable in the precipitate when redissolved. This may possibly be due to the occurrence of such a tautomer. The analogous mono-acid salt of safranine, violet in colour, occurs in solutions of moderately high acidity.

Confirmatory evidence of this hypothesis is available. The above reactions would necessitate the enzyme or combining substance to be predominantly acid in character. That this is so, is inferred from the experiment of Bayliss [1906] who showed that trypsin migrated to the anode when subjected to the influence of an electric current. This would be expected if the trypsin were acidic in character. The complete loss of the ability to precipitate the enzyme by the reduced "leuko" compound further supports the hypothesis of its linkage to the azine N.



The enzyme or the substance which carries down the enzyme so completely is precipitable by the azine and azonium bases, the basic N of the azine nucleus being active in this precipitation.

The study of the action of the azine bases was extended to other enzyme preparations. By means of fractional precipitation with alcohol and subsequent dialysis, very active preparations of trypsin may be prepared from extracts of the pancreas. However, the proteolytically active substances, precipitated by the azine bases from these so-called "pure trypsin" preparations, constitute but a very small proportion of the whole material, rarely more than 5 %. No preparation was examined from which the proteolytically active substance could not be precipitated quantitatively by the azine bases.

The azine precipitates from crude gland extracts retain their proteolytic activity, but show no lipolytic or diastatic activity, although both of the enzymes responsible for the latter actions may be demonstrated with ease in the original glandular extracts. The experimental data of this work will be published in a later paper.

The substance precipitable by azine retains the power to clot milk. As this property is common to all the proteolytic enzymes, the action of the azine bases was tried towards proteolytic enzymes from other sources, with the remarkable consequence that in all cases the azine base completely precipitates the whole of the proteolytically active substances from their solutions, thus:

Enzyme	Source	Base	Activity of azine	
			ppt.	pH
Pepsin	Stomach extract (pig)	Pheno-safranine	+	2 (HCl)
Trypsin	Pancreas (pig)	"	+	7.5
Erepsin	Succus entericus (pig)	"	+	7.5
Papain	Paw-paw (Parke, Davis and Co.)	"	+	5.0
Erepsin	Yeast	"	+	7.5

Fibrin was used as substrate in the case of the pepsin and trypsins; "Difco" standard peptone being used for the demonstrations of the activity of the erepsins.

Separation of Pepsin from Azine-pepsin Compound.

Owing to the stability of pepsin towards acids the pepsin-azine compound was further studied. At pH 1.2 the compound is decomposed, and the base may be extracted with amyl or butyl alcohol. When the compound is shaken in a separating funnel with 0.5 % HCl and butyl alcohol, and allowed to stand, three layers are formed. The butyl alcohol layer contains most of the azine. A stable emulsion of butyl alcohol constitutes the second layer. The third or watery layer which should contain the free enzymes, however, is inactivated by this process. The active constituent was found to be adsorbed at the surface of the butyl alcohol droplets in the emulsion. When this emulsion is broken up by the addition of a few drops of ether, the original proteolytic activity is regained.

The pepsin-azine precipitate has been prepared in quantity. It is not widely different in its physico-chemical behaviour, from the trypsin-azine

precipitate. However, it is soluble in 0.5 % HCl. The effect of the quantitative precipitation of the proteolytically active substance from solution by the azine bases may be easily demonstrated with pepsin solutions because of their relative stability.

DISCUSSION.

The power of the azine and azonium bases to remove completely the proteolytic enzymes from solution indicates that a direct union of the enzyme and the precipitating base is established. The linkage has been shown to occur through the basic nitrogen of the heterocyclic azine ring; this union, instituting a tautomeric rearrangement within the azonium base, causes a colour change. The recovery of the proteolytic power of the azine-enzyme compound depends on the conditions which most favour its dissociation. The compound is more highly dissociated by an increase of H ions. At p_H 2.0 the pepsin-azine compound, for example, is decomposed and the red azine dye set free.

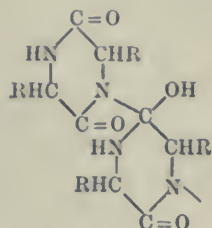
The compound is but slightly soluble in water at neutrality, but complete solution is obtained by the addition of peptone or protein digestion products. This, in all probability, is due to the dissociated enzyme ion combining with the protein digestion products, liberating the azine which reverts to the ortho-quinoid structure, the whole compound thus being decomposed and carried into solution.

The combination of the proteolytic enzyme with the azine nitrogen gives us evidence as to the nature of the combination between this enzyme and its substrate.

From time to time in biochemical literature the occurrence of diketo-piperazines in protein digests has been demonstrated. The presence of leucine anhydride among the cleavage products was first mentioned in 1849 by Bopp [1849]. Salaskin [1901] isolated diketo-piperazines from peptic and tryptic digestion products of oxyhaemoglobin. Abderhalden [1903] showed the presence of about 1 % of leucine anhydride in the products of HCl hydrolysis of caseinogen. He suggested that this anhydride was produced as a secondary product of leucine, and attempted, unsuccessfully, to demonstrate the reversal of the reaction. Water hydrolysis of proteins at high temperature produces about 1 % of leucine anhydride [Graves and Marshall, 1917].

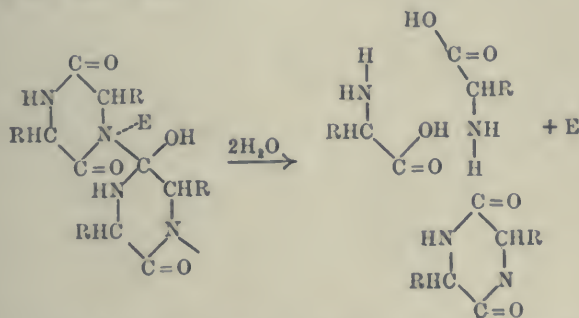
The catenary or chain-like configuration, as accepted in modern protein literature, fails to explain many peculiarities of the chemical actions of proteins. Such a structure would necessitate at least one free amino group at the end of the molecular complex. However, contrary to this, Van Slyke and Birchard [1914], and others, have shown that the nitrogen liberated from native proteins by the action of nitrous acid is equal to one-half of the lysine nitrogen content, that is, it is liberated from the NH_2 groups of the lysine bound up in the protein molecule. Some proteins, salmin and zein for example, when pure do not yield any nitrogen with nitrous acid, thus demonstrably lacking any free NH_2 groups.

These facts, together with other evidence hitherto unpublished, have led the author to put forward the hypothesis of a new form of protein structure. Instead of the conventional structure, the protein molecule may be considered to be built up of a series of amino-acid anhydrides, or, in other words, protein has essentially a poly-diketo-piperazine structure. Taking the type amino-acid $R.CH(NH_2).COOH$ for example, the protein complex is represented by this type of structure as



Such a structural configuration of the protein molecule would make quite clear the absence of terminal NH_2 groups. Peculiarities of protein ionisation and their neutralising power may be easily explained by means of this suggested structure. The similarity of each ring to the azine nucleus of the bases which are functional in combining with the enzyme suggests that the N in these groups is the seat of action of the enzyme.

The proteolytic enzyme E, combining with the nitrogen (N) of the diketo-piperazine ring would necessitate a structural change to supply the fifth valency of the nitrogen atom.

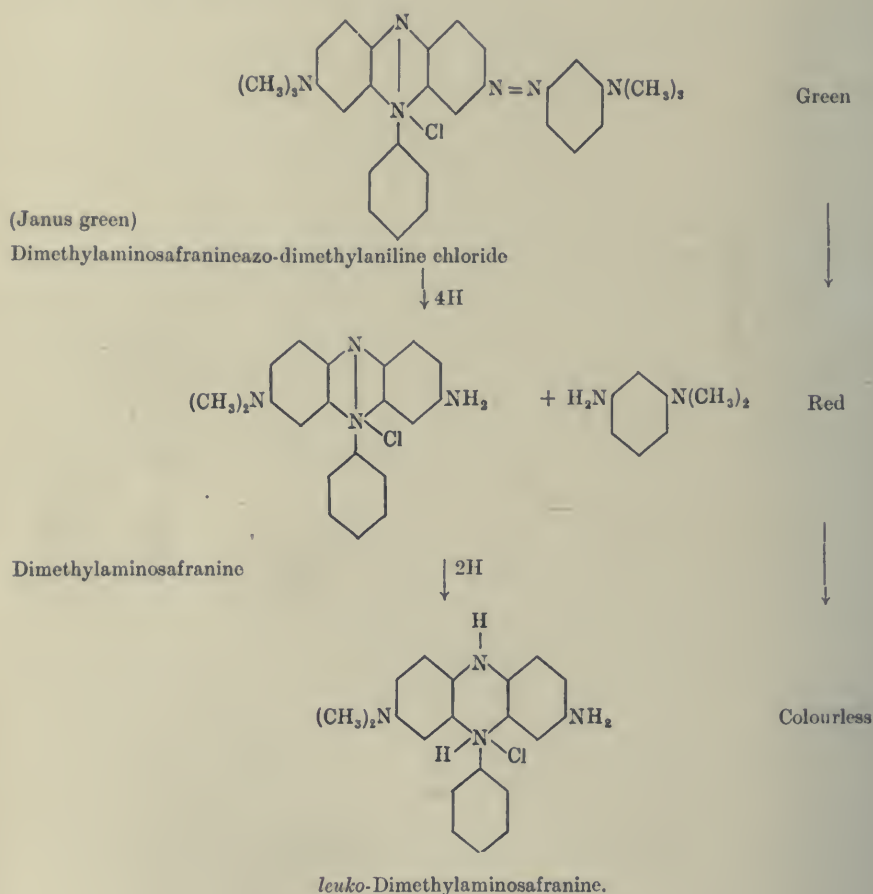


This could be satisfied by the addition of two molecules of water, resulting in the separation of two molecules of amino acid. The enzyme at this point is set free to act on a fresh group.

As the azine and azonium bases are coloured substances the occurrence of the proteolytic enzymes in the cell may be demonstrated by means of their selective staining reactions.

Michaelis, in 1899 [quoted by Cowdry, 1918, p. 86], while making a detailed study of the behaviour and chemical nature of the *intra vitam* dyes, found that dimethylsafranineazodimethylaniline (Janus green) specifically stained certain structures in the living cell. These structures have since been demonstrated

in all cells, from the bacterium to the angiosperm in the plant kingdom, and from amoeba to man in the animal kingdom. Cowdry [1918] showed that ethylsafranine will also stain specifically the mitochondria (as these structures are generally named). He also demonstrated that the preparation stained with Janus green, if kept under anaerobic conditions, will manifest a series of colour changes, the mitochondria changing from green to red and then becoming colourless. This, he points out, is due to the reduction of the dyestuff. If we follow out the reactions involved



we see that the point of combination of the dye with the mitochondria could be no other than the N of the azine nucleus. After the stained tissue preparation is bleached by reduction it cannot be restained by application of more dye, so the groups to which the dye is linked are fully saturated with the dye. The modification of the mitochondrial constituents of the cells into zymogen granules during active secretion of the pancreas has been demonstrated by various workers.

The specific staining of the mitochondria with the azine dyestuffs indicates that the proteolytic enzymes are concentrated in these bodies. The mitochondria may be thus the site of syntheses in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favourable conditions for the synthetic activities of the enzyme.

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Note.—The possibility of the existence of diketopiperazine rings in the protein molecule is discussed by Abderhalden [*Z. physiol. Chem.* 1923, **128**, 119] in a paper which appeared in May 1923 but was not accessible in Adelaide at the date when Mr Marston's paper was despatched to England.
 EDITOR.]

CVI. SOME EXPERIMENTS AND REMARKS ON THE POSSIBLE TRANSFORMATION OF *d*-GLUCOSE IN THE INTESTINE AND ON THE NATURE OF BLOOD-SUGAR.

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In this article are communicated the results of experiments on carbohydrate-metabolism, which are closely connected with those recently published by a number of English investigators. These experiments deal, namely, with the possible transformation which the stereoisomeric glucose-forms, present in a solution of *d*-glucose in equilibrium, undergo in contact with the intestinal mucosa, and with the nature of blood-sugar.

I began these investigations at the suggestion of Prof. Hamburger as early as July, 1922. At first it was not my intention to communicate the results of these experiments yet, though their number in both problems was a large one. However, as I shall not have opportunity to continue my experimental work on these and allied subjects in the nearest future, I give here a short account of my work, especially as I am informed that the publication of the results reached up to the present may be of value for others, and also, as in more than one respect the methods used are different from those followed by the English investigators.

The reason for undertaking the experiments on the subjects mentioned, was the results of the investigations of Prof. Hamburger and Dr Brinkman on the permeability of the kidney towards isomeric and stereoisomeric sugars, which were published in different papers [Hamburger, 1919, 1, 2; 1921, 2; 1922, 1; Hamburger and Brinkman, 1918, 1], and discussed by Prof. Hamburger in two lectures [Hamburger, 1921, 1; 1922, 2]. The results of these investigations seemed to prove that the permeability of the kidneys for the stereoisomeric forms of the same sugar could be quite different.

Of these numerous experiments I wish to draw attention here only to those concerning the partial retention of the kidney for *d*-galactose [Hamburger, 1921, 1, 2; 1922, 1, 2], which was always about 50 %. Therefore Hamburger concluded that the only reason for this partial retention could be the fact, that of the two modifications of *d*-galactose in solution, namely the α - and the β -variety, one form is retained by the kidney, the other form is not held back.

Similar results were obtained with the two varieties (α - and β -) of another mutarotatory sugar, *l*-xylose. Here 25 % was always retained.

To study the mechanism of this difference, a number of experiments were made. In addition the question was put whether the difference in permeability, found between the isomeric and stereoisomeric forms of the same sugar, might also have a practical significance for the diabetes problem. That means, the question was introduced by Hamburger, whether the quite different behaviour of the normal and diabetic organism towards glucose had as underlying principle a stereoisomeric difference between the glucose of the blood under normal and pathological conditions [Hamburger, 1922, 2].

In the literature we find a few examples of a different behaviour of the organism—of the normal as well as of the diabetic—towards the stereoisomeric forms of the same sugar. Thus a difference was found in the behaviour of the human body towards different stereoisomeric methylglucosides [Lit. cited by Neuberg, 1911]. But that really in the normal organism one special stereoisomeric form of glucose is preferred, seemed to be demonstrated by the well-known experiments of Hewitt and Pryde [1920]. They seemed to indicate with some certainty, that normally by the resorption of carbohydrates in the intestine these are not only all transformed into glucose, but principally into one definite stereoisomeric form of glucose of a very reactive nature and with a negative or weak positive specific rotation (γ -glucose?). They would also show us that we might expect the possibility of the presence of such a reactive sugar, which is in equilibrium with *d*-glucose, in the blood. It is well known to the readers of this *Journal*, that in the recent investigations of Winter and Smith [1922; 1923, 1, 2, 3, 4; also Forrest, Smith and Winter, 1923] the existence of such a reactive sugar in the normal blood is assumed to be very probable.

As both questions, the transformation of *d*-glucose in the intestine and the nature of the blood-sugar, were fundamental and might be closely connected, Prof. Hamburger advised me to take up the problem of the stereoisomeric sugars. At his suggestion a beginning had already been made with a repetition of the experiments of Hewitt and Pryde. I have continued and extended these experiments.

In the first series of experiments in our laboratory only narcotised rabbits were used and aqueous solutions of very pure glucose in equilibrium of a strength of 2 to 3 %.

As the results reached in these experiments wholly correspond to those just published in detail in this *Journal* by Stiven and Reid [1923], I may refrain from a detailed statement of our results. This especially as our technique, as regards filtration and care for accurate polarimetric readings, largely corresponded to that followed by these authors.

About the procedure I only wish to say, that we worked so quickly, that the first polarimetric readings of the clear filtrate were always made within three minutes after the liquid had been removed from the intestine. The polarimeter which we used, in these and the following experiments, was a two-field instrument with which readings to a maximal accuracy of 0.01° could be made. The readings in these experiments had in nearly all a maximal error of 0.04°. The solution remained in contact with the mucosa from 5-15 minutes.

For washing the loop of intestine before it was filled with glucose, we used in two experiments Tyrode's solution made up without glucose, and in four experiments a modified Ringer's solution of the following composition: NaCl 0.7, KCl 0.01, NaHCO₃ 0.2, CaCl₂ 6 aq. 0.02 %.

Before the beginning of the experiment we passed CO_2 through this solution till $[\text{H}^+]$ was $0.45 \cdot 10^{-7}$ (determined with neutral red as indicator). In this way we had a solution of which the $[\text{H}^+]$ and $[\text{Ca}^{++}]$ correspond to those of the serum of the animal used, and which, from the well-known experiments on the retentive power of the kidney for sugar [Hamburger, 1919, 1, 2; Hamburger and Brinkman, 1918, 2], and from those on the resistance of the red corpuscles [summarised by Hamburger, 1922, 3], may be regarded closely to approach the normal physiological fluid.

I give here as illustration only the shortened report of one experiment.

Exp. A. Rabbit, weight 2500 g. No food was given in the last 24 hours before the experiment began.

Morphine-anaesthesia; 60 mg. HCl-morphine intravenous.

Glucose solution of about 2 %, prepared 18 hours before the beginning of the experiment; rotation in 20 cm. tube $2^{\circ}04$ (mean of six determinations).

Beginning operation five minutes after morphine injection.

Intestine loop used, directly behind stomach, of about 40 cm. length, tied off, two canulae in the ends, washed out with Ringer fluid at body-temperature, then with glucose solution and filled with the latter carefully. Glucose in intestine from 10 h. 30–10 h. 44. At 10 h. 44 content of intestine loop filtered and filtrate investigated in 10 cm. tube.

10-45	+1.30°	10-54	+1.28°
10-48	1.32	11-07	1.26
10-49	1.26	11-21	1.26
10-51	1.29		

No alteration after addition of a trace of alkali.

It is seen, that in this case the first reading (in the table is always given the average of several readings) was made within one minute after the liquid had been removed from the intestine. The difference between the first and the following readings was in this and also in the other cases within the limits of permissible error.

Altogether six of these experiments with narcotised rabbits have been done, but like Stiven and Reid, who have done a much larger number of experiments, we have in no case obtained results of the same nature as those of Hewitt and Pryde.

In two of the six experiments we determined the reduction of the filtrate immediately and also after a few hours, without finding a difference of importance. Compared with the polarimetric value, the reduction, determined by Bang's method, was constantly a little higher.

We have repeated these experiments under conditions which in my opinion may be called more physiological than those with the narcotised rabbits; namely, we made use of a dog, in which I had made a *Vella-fistula* of the small intestine (the length of the intestine loop was about 40 cm.). With this dog I have repeated the experiments of Hewitt and Pryde, now of course without anaesthesia, at least 25 times, but, as may be said directly, also with negative result.

In these experiments the fistula was first washed out with the usual Ringer or Tyrode, heated to 37° . We then injected the 2 % solution of glucose in equilibrium, also heated to 37° , and at a certain moment, whilst one person injected the solution slowly into one end of the fistula, another closed the other opening with his fingers. Then the injection-canula was removed and the other opening also closed with the fingers. The dog was a very quiet one and with the openings of the fistula firmly closed with the fingers, remained standing for the whole length of time that the fluid remained in the intestine, i.e. for 5–10 minutes. Generally about 50–60 cc. of the glucose solution was in the intestine loop at the moment it was closed. At the end of the determined time we could not wait till a peristaltic action of the bowel removed the unresorbed liquid and therefore it was washed out with a quantity of the modified Ringer or

Tyrode. The liquid obtained was filtered directly and the clear filtrate investigated in the polarimeter tube at a temperature of 37°, although in the first four experiments we made the readings at room-temperature just as in the preceding experiments. The observation-time in the experiments with the dog was much longer than in those with the rabbits.

I give here as illustration a shortened report of one of these experiments, which may serve as a good example of the general results obtained in all. It is seen that also in this experiment we did not get results similar to those obtained by Hewitt and Pryde, though the conditions were, I believe, much more physiological.

Experiment 1 with fistula dog.

Glucose solution of about 2 % in equilibrium. Fistula loop washed out with Tyrode, made up without glucose.

3.21-3.29 glucose solution is in the fistula.

3.29 fistula is washed out with Tyrode.

Polarimetric readings in tube of 10 cm.

3.315	+ 0.74°	3.48	+ 0.70°
3.325	0.70	3.485	0.70
3.345	0.72	3.495	0.67
3.37	0.70	3.53	0.70
3.385	0.68	4.08	0.72
3.40	0.68	4.09	0.71
3.415	0.66	Next morning at 9.15 + 0.68.	
3.44	0.67	After addition of trace of alkali no	
3.45	0.68	change.	

After we had read the experiments of Winter and Smith, which will be shortly referred to later on, we thought of the possibility, that *insulin* might play a rôle in the transformation of glucose in the normal intestine and that insulin was absent from the mucosa of the fistula loop. We have therefore, in four experiments with the fistula dog, added a trace of insulin to the glucose solution in equilibrium brought into the intestine, although our experiments in this direction with rabbits had also given negative results. However in these experiments we again did not get indications of mutarotation.

In the experiments with the dog we also very often compared the reduction and polarimetric value of the filtrate directly (within a few minutes after the clear filtrate had been obtained) and after one or two hours, once also after 24 hours. Here again we found no difference of importance.

Although I have often tried by altering the details in the various experiments (small variations of the p_{H} in the modified Ringer used, etc.) to get a positive result, that is to say, to observe that the liquid obtained from the intestine showed an upperwards tending mutarotation of importance; yet, as already mentioned, and to my regret, like Stiven and Reid, I was never able to manage it, let alone to get a negative reading such as Hewitt and Pryde reported they had obtained once.

In the meantime we had also commenced to study the problem of the nature of the glucose in the circulating blood, since dealt with fully by Winter and Smith [1922, 1923, 1-4; see also Forrest, Smith and Winter, 1923]. We expected from the beginning that if the blood-glucose consisted mainly of one stereo-isomeric form of glucose and that if we tried to investigate this by using blood itself, there was reason to expect a rapid change to the α - β -equilibrium; as the

blood had to be deproteinised before the reduction or optical rotation could be determined, which takes time. Then, if the deproteinisation is done chemically, this in itself might have an influence and invalidate the experiment¹. Looking for other possibilities, we thought of the *aqueous humour* and of the *serum ultrafiltrate*; the first of these had been previously used in researches of this laboratory several times [van Creveld, 1921; de Haan and van Creveld, 1, 2].

For the problem in question, the aqueous humour, which may be regarded as an ultrafiltrate or better as a *vivo*-dialysate of the blood-plasma, has several advantages: it is colourless, water-clear, does not clot (the primary liquid at least) and contains only a trace of protein. It is therefore possible to determine reduction and polarimetric value of the liquid of the eye-chamber for the first time directly after puncture. A drawback was of course that the amount of glucose in the normal chamber liquid is only small, smaller than in the blood-plasma [de Haan and van Creveld, 1921, 1, 2]. Notwithstanding this disadvantage we thought it to be of great importance to see in how far with the eye-chamber liquid, which was not treated in any way, indications about the stereochemical forms of glucose in the blood under normal and pathological conditions might be obtained. We made the puncture of the cornea in the same way as in former researches with aqueous humour, namely with a sharp-pointed glass capillary, and in the upper part of the cornea, after cocainisation with 2 % HCl-cocaine. From each rabbit's eye about 0.3 cc. may be obtained.

First we looked for a qualitative test of the presence of a definite stereoisomeric form of glucose. As such the *decolorisation of permanganate* may be regarded, as it may point to the existence of a reactive sugar. We compared the rate of decolorisation of a 1/100 *N* weak alkaline solution of potassium permanganate by the eye-chamber liquid at different times after the puncture.

In these experiments with normal animals we found that, whereas *the addition of the eye-chamber liquid immediately after the puncture caused a rapid and intensive decolorisation of the permanganate*, this became slower the longer the time that elapsed between the time of puncture and the moment of addition to the permanganate. Using a proportion of 2 cc. *N*/100 KMnO_4 to 0.2 cc. eye-chamber liquid I found that after 25 minutes to half an hour, the rapidity of decolorisation had decreased in a high degree and was then not much larger than that of a 0.5% solution of glucose in equilibrium, used for comparison. There is a possibility that the rapid decolorisation of permanganate by the fresh eye-chamber liquid is due partly to the presence of auto-oxidisable substances. It is difficult, however, in my opinion, thus to explain the whole decolorisation. The relatively slow decrease of the power of decolorisation is in any case difficult to account for in this way and it is also of importance to mention at this point that the reduction and the refractive index of the eye-chamber liquid, determined at different moments during the first hour after the puncture, appeared not to decrease. The change in the behaviour towards permanganate might thus surely be in favour of the presence of a reactive sugar in the normal aqueous humour.

To find out in how far other findings were in accordance with this, we investigated the polarimetric value of the aqueous humour during the first hour

¹ Compare here also the remarks of Hewitt [1923].

after the puncture. Here we made use of the micropolarisation method as described by E. Fischer [1911]. This was necessary, as the results with the permanganate test seemed to us to indicate that if the eye-chamber liquid showed mutarotation, the changes might take place during the first half-hour after the puncture. We therefore worked as quickly as possible, using only a small quantity of liquid, obtained from one or two eyes. Waiting till we had collected sufficient liquid from several rabbits to fill a common polarimeter-tube would have taken too much time.

Making use of the figures obtained in a former research, concerning the average sugar-content of the normal aqueous humour of the rabbit, we calculated whether the possible mutarotation of this liquid could be determined with sufficient accuracy. We found that, accepting that the specific rotation of the greater part of the glucose present in the eye-chamber liquid was low and that outside the body a specific rotation of 52.5° could be reached, we then should need at least a 10 cm. tube to be able to investigate the existence of a mutarotation accurately enough, although we should be unable to determine this quantitatively. For technical reasons, tubes longer than 10 cm. cannot be used for micropolarisation. Therefore we lack the ability of reading off a greater rotation and herewith the chance of determining a possible mutarotation with greater nicety.

The tubes were filled with the aqueous humour by means of a glass capillary, as soon as possible after the puncture of the cornea had been made. The first readings were usually made within one or two minutes after the punctures. All readings in these experiments were at room temperature. My eyes, as well as those of the persons who were kind enough to control my readings by making these readings by turns with me, were trained in looking through the polarimeter with the capillary tubes in it. The reading error with these tubes was, when we were well adapted, 0.02 – 0.03° .

I have done a large number of these experiments with the aqueous humour of normal rabbits. At first we only made as many readings as we could during the first half-hour after the puncture and then took a few readings after a drop of alkali had been added. In later experiments we continued for some hours to make readings at intervals of a few minutes. Because of the great number of such experiments we came to the conclusion, however, that the sugar content of the normal aqueous humour is so small that a possible mutarotation, even qualitatively, could not be determined by this method. We believe, it is true, that we have found with tolerable certainty a few times (in five experiments) that the optical rotation found during the first 5–15 minutes greatly increased, sometimes even to more than 100 % of the original value. That increase differed, however, too little from the reading error which we had fixed. This is possible, as the rotation—not of course the same in all experiments—amounted to an average of only 0.08° . We have never found a pronounced negative original rotation, though we found a few times a value of 0.00 – 0.02° , which apparently increased to 0.04 – 0.06° . Comparison of the reduction with the optical rotation has, within the bounds of the reading error, always shown a fairly good agreement. The addition of a trace of alkali to the aqueous humour brought about no distinct alteration in the optical rotation.

It occurs to me, that the doubtful result of the experiments mentioned

above need not be a reason why the use of aqueous humour for an investigation on the nature of glucose in the blood and especially of the stereochemical modifications should be given up. If it were possible to determine the optical rotation of the aqueous humour with greater exactness than is now possible, *e.g.* by using a polarimeter with an objective reading, then the doubt which, I must conclude, now exists concerning the mutarotation of the normal aqueous humour would surely be done away with. Perhaps the optical rotation of the aqueous humour might then also be determined *in vivo*.

The above mentioned investigation of normal aqueous humour was accompanied by a similar investigation of *diabetic* aqueous humour. For this we chose the *aqueous humour after adrenaline injection*. It is shown from the experiments carried out by de Haan and myself [1921, 1, 2] that the hyperglycaemia after subconjunctival adrenaline injection, shows itself only slowly in the aqueous humour, though the increase of the sugar content therein is also very great. In consequence of the results obtained in these experiments, I have now investigated in a number of cases the aqueous humour, two hours after a subconjunctival injection of about 0.75 cc. adrenaline, 1/1000. We also made a qualitative examination of the behaviour towards an alkaline solution of permanganate.

We expected a less rapid decolorisation in connection with the supposition that the diabetic sugar is chiefly a different (stereochemical) modification of glucose than the normal, and with the fact that the behaviour of the normal aqueous humour towards permanganate, as described above, seemed to indicate the presence of a reactive sugar in this liquid. It appeared, however, that *the same permanganate solution became decolorised much more quickly and more intensively by the aqueous humour of the adrenaline injected rabbit than by the normal aqueous humour*. Twenty minutes after the puncture the difference was still clearly to be seen. This difference is probably not to be explained simply by the raising of the sugar content of the aqueous humour, as might be deduced from the control experiments with glucose solutions at similar alkalinity. May we, then, perhaps assume that an equilibrium α - β -glucose \rightleftharpoons reactive sugar (γ -glucose?), existing (as supposed by Winter and Smith) normally *in vivo*, is changed after adrenaline injection in the direction of the reactive sugar? The latter would not be in accordance with the hypothesis of Winter and Smith.

The polarimetric investigation, compared with the results of the reduction analyses, might possibly teach us something about this.

Before mentioning the results of such investigations, I wish to draw attention to the fact, that in the literature a test has already been described for distinguishing normal from diabetic blood, which also depends on the reducing properties of the blood (aqueous humour). This test is Williamson's test [Cambridge, 1913]. According to this, methylene blue in alkaline solution is decolorised by diabetic blood (and also by diabetic urine), but not by normal blood. This test, although not wholly in agreement with the difference found in behaviour of normal aqueous humour and this liquid after adrenaline injection, still points in the same direction as regards a possible quantitative proportion between α - β -glucose and the reactive sugar in the normal and diabetic blood.

The result of the polarimetric investigation, compared with the results of the reduction analyses of the eye-chamber liquid after adrenaline injection, have, however, in a large number of experiments, supplied no arguments at all in favour of such an hypothesis, and thus far are more consistent with what Winter and Smith find probable for diabetic blood. The optical rotation now remained, with only a single exception, within the limits of the reading error and this remained so from the moment of the first reading during some hours, after which observations were discontinued. Addition of a trace of alkali did not alter the optical rotation.

The increase of the sugar content, determined by the reduction method, was important in all experiments and in connection herewith also the optical rotation which corresponded closely with the reduction value. The average rotation was $+0.22^\circ$. A possible mutarotation of the same nature as formerly supposed might thus have been determined in every case with greater accuracy than in the normal aqueous humour.

The question arises whether any importance as regards a difference in the glucose modification, may still be ascribed to the difference found in the behaviour towards the permanganate of normal and of diabetic aqueous humour. Of course it is possible that disturbance of the equilibrium suggested above, really takes place, but that this is of such a nature, that it cannot be determined polarimetrically. However it may be, these observations teach us again, that one cannot be too careful in drawing conclusions concerning the nature of the glucose modification from the behaviour of the fluid under investigation towards permanganate.

After the experiments on the nature of blood-sugar, described above, were completed, the first publications of Winter and Smith on the same subject came to our notice. The results obtained by these investigators induced me to extend the experiments. The authors have compared the reduction and the polarimetric value of a concentrated protein-free glucose solution, obtained by special technique—which involves a chemical deproteinisation—from blood, under normal and diabetic conditions. In normal men and animals they found that, whereas the reduction of the solution during some days remained constant, the optical rotation increased in three to four days from an original low value, to a constant value, which corresponded with the specific rotation of the α - β -glucose and with the reduction of the solution.

On reading these investigations, we were struck at once by the *slow* change found by Winter and Smith: the equilibrium was only reached after some days. The possibility of such a slow alteration had never been obvious in examining the aqueous humour. This could, however, be connected with the small rotation of the normal aqueous humour that was only examined in a 10 cm. tube. We have therefore examined greater quantities of aqueous humour in ordinary polarimetric tubes of 20 cm. length, and we have extended the time of observation over 4–5 days. Thereupon, we examined *aqueous humour* (in tubes of 10 or 20 cm. length), *of which the glucose concentration was increased by concentration of the liquid in vacuo*. And, thirdly, we have extended our research to *ultrafiltrates*—either concentrated or not—*of blood serum and of artificial transudates*.

The greater quantities of eye-chamber liquid were obtained either by puncture of the eyes of several rabbits or from cows' eyes, brought fresh on ice from the abattoir.

For the ultrafiltration a large apparatus of Bechhold was used, with collodion filters of 3 % or 6 % strength. The artificial transudates were obtained by injecting 100–200 cc. NaCl 0.9 % or modified Ringer's solution, of which the composition was given above, into the peritoneal cavity and drawing off the unresorbed liquid, which now contains sugar, after some hours¹. From this liquid, one obtains a large amount of ultrafiltrate much quicker than from blood serum, and with about the same sugar content as the serum-ultrafiltrate possesses.

The advantage of using a concentrated ultrafiltrate above that of the concentrated aqueous humour lies herein, that the first-named can be kept absolutely free from protein, whilst by the latter with the increase of the sugar-content the trace of protein may also increase to a disturbing concentration. The advantage of the deproteinisation by ultrafiltration over the chemical deproteinisation as followed by Winter and Smith for the subject in question cannot be denied.

Of the various liquids here named we now compared the reduction and optical rotation for some days, and in a large number of experiments. During the time between the several observations the liquids were kept at low temperature.

I have been unable to obtain with the liquids I have used similar results to those which Winter and Smith described for the concentrated protein-free filtrates prepared from normal blood. Time and again I have found that the reduction of the examined liquids remained the same for 4–5 days and that there was also no alteration in the rotation. There was always a small difference between reduction and optical rotation in favour of the former.

We have also had the opportunity of testing an hypothesis suggested by Winter and Smith in connection with their own experiments and with those of other investigators. They have, namely, expressed the possibility that only the glucose form which in normal men and animals forms the greater part of the blood-sugar (the reactive sugar with a small positive or negative rotation) may be used in metabolism. Further, that in the production of this reactive form, insulin may play an important rôle. It seemed to us that the experiments made by Hepburn and Latchford in Prof. MacLeod's laboratory [1922] formed suitable material from which to get support for that hypothesis, and indirectly also for the nature of blood-sugar. These investigators have found, namely, that in perfusing the mammalian heart by Locke's method [Locke and Rosenheim, 1907], the addition of insulin to the perfusion-fluid caused a quantity of glucose to be withdrawn from the perfusion-liquid, which was four to six times as large as the normal quantity. In how far was this due to an increased formation of the so-called reactive form of glucose? We felt justified in putting this question after the well-known experiments of Clark [1916] and others, and of Winter and Smith themselves, although it was not proved that there is actually an increased sugar-consumption under influence of insulin.

Like Winter and Smith, I had found in *in vitro* experiments that insulin alone has no influence on the optical rotation of *d*-glucose in equilibrium. It was possible, however, that the effect found by Hepburn and Latchford was

¹ Compare Feringa [1922].

due to a co-operation of insulin and heart-enzyme *in vivo*, which resulted in an increased transformation of the glucose of the perfusion liquid into the reactive form. If such a process did not take place intracellularly, it might possibly be determined by investigating the perfusion liquid issuing from the heart.

In the perfusion apparatus, at a short distance under the heart, we have therefore fastened a T-piece, through which at any moment a quantity of liquid coming straight from the heart, could be obtained. The reduction and the optical rotation of this liquid were determined at 37°, with or without filtration through a Gooch filter. (When the heart-perfusion lasts somewhat longer, the perfusion liquid always becomes opalescent, as Locke has stated already.)

In the first two experiments, in which no insulin was added, it appeared that the liquid coming from the heart and investigated within one or two minutes afterwards, did not show mutarotation. Then we have in five other experiments, after the perfusion had lasted about one hour, added to the perfusion liquid bit by bit a small quantity of insulin, dissolved in a little perfusion liquid. In three experiments we have found distinctly an increase of the amount of glucose withdrawn from the perfusion liquid, as compared with the normal condition. In no case, however, did the issuing liquid show any mutarotation, either spontaneously or after addition of alkali.

How favourable were the conditions under which these experiments were carried out, may be seen from the following. We thrice observed, that after the heart had contracted regularly for about eight hours and then had stopped beating after the O₂-supply and the warming of the perfusing liquid had been stopped, and was left for about 20 hours at room-temperature, yet the heart began again to beat rather strongly, when the O₂-supply and the warming of the perfusion-liquid were again set going.

DISCUSSION.

In considering the experiments on the nature of blood-sugar described above and judging from these and comparing them with those of other investigators, I must conclude that the question of the stereochemical forms of glucose present in blood under normal and pathological conditions is not yet settled at all. I obtained in my experiments only very small indications about the existence of definite stereoisomeric forms of glucose in normal and pathological blood. Notwithstanding this, the principle of the question, namely, that in blood different stereoisomeric forms of glucose occur with a different biochemical significance, in our opinion need not be given up. The results obtained in those biochemical experiments in which more or less pure solutions of the stereoisomeric sugars were used, such as those of Hamburger [1919, 1, 2; 1921, 1, 2; 1922, 1, 2] and of Hamburger and Brinkman [1918, 1], and the investigations recently published by Willstätter [Willstätter and Sobotka, 1922] give, in our opinion, much support to this conception. That in the case of blood it is so extremely difficult to give direct proofs, may be partly due to technical difficulties, which might be overcome in future, and of which I could mention some.

Then we must remember that the chemical side of the problem is still very complicated. I tried from the beginning to get information about the sugar

of blood principally by comparing the reducing and polarising power of the liquids used, just as had been done by Winter and Smith. Our idea was that the polarimetric evidence alone would be sufficient. This has since been doubted, especially as far as the detection of the so-called γ -sugars is concerned, from the side of the chemists, whereas they also think it highly probable that γ -sugars play an important rôle in carbohydrate metabolism. If their conception is right, then of course we cannot expect to get more information about the presence of γ -sugars in blood as long as we do not know more about the properties and the detection of these sugars in the organism.

The difficulties also concern the question of the mechanism of the action of insulin, if it becomes more probable that this really is connected with a conversion of the stereochemical forms of glucose into each other. Until now, however, this question also seems not to be settled [MacLeod, 1923].

I hope that if the conditions are more favourable in every respect for further research, the methods and results described here may appear to be of some value in obtaining more information about the nature of the normal and diabetic blood-sugar.

SUMMARY AND CONCLUSIONS.

1. A repetition of Hewitt and Pryde's experiments on the transformation of *d*-glucose in the intestine, with narcotised rabbits and with a dog in which a Vella-fistula was made, has not given any evidence for stereochemical changes of the nature described by these authors.

2. To study the nature of blood-sugar, *aqueous humour* and *serum-ultrafiltrate* have been used. Investigated during the first half-hour after it had been obtained from the eye, the *normal* aqueous humour showed a rapid decolorisation of permanganate directly after the eye-puncture, which decreased in 20–25 minutes in a very important degree. With the technique used, a possible mutarotation of the normal unconcentrated eye-chamber liquid, even qualitatively, could not be determined with certainty.

Reduction and optical rotation of the normal eye-chamber liquid showed a fairly good agreement.

3. After (subconjunctival) adrenaline injection the rapidity of decolorisation by the aqueous humour is increased. With great certainty it may be said that after adrenaline injection the aqueous humour does not show mutarotation of any importance and that the optical value under these conditions closely corresponds to the reduction value.

4. Reduction and optical value of serum-ultrafiltrate, of concentrated serum-ultrafiltrate, of aqueous humour and of concentrated aqueous humour, compared during four to five successive days, may remain the same from the beginning of the experiment, and closely correspond with each other.

5. During the increased removal of glucose from the perfusion-liquid of the heart, observed when insulin is added, no sign of mutarotation can be observed in the issuing liquid, which could point to the formation of a "reactive" sugar.

In conclusion it gives me great pleasure to thank Prof. Hamburger for his constant stimulating help, criticism and advice in the course of these investigations.

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CVII. THE ALLEGED SPECIFIC COLOUR REACTION FOR THE ANTISCORBUTIC FACTOR.

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(Received October 8th, 1923.)

THE disadvantages which attend the biological methods employed in the detection of the vitamins have always kept in the foreground the necessity of devising a chemical means for their estimation. As long as the accessory factors remain chemically unidentified principles it is only possible to fall back on indirect methods, such as colorimetric methods, of estimation.

Funk and Macallum [1913] have demonstrated a certain parallelism between the colour reaction with the Folin and Macallum reagent for the determination of uric acid and phenol and the antineuritic activity of some products. Similarly, Drummond and Watson [1922] have drawn attention to the striking association of the fat-soluble A factor with a substance in oils giving the well-known purple coloration with sulphuric acid. Neither of the above reactions has, however, been found reliable enough to replace the biological methods of estimation of these vitamins.

Recently Bezssonoff [1922] devised a reagent—a modification of Folin's reagent for phenol—which he claimed gave a specific colour reaction for the antiscorbatic factor, because he found that a number of substances known to be antiscorbatically active produced a positive reaction with this reagent.

By employing this reaction on a great number of substances which have been simultaneously tested out on guinea-pigs we have come to the conclusion that, although there is a certain association between this colour reaction and the antiscorbatic activity of some substances, the relationship does not hold true in all cases. We have been able to find, on the one hand, some antiscorbatically active substances which fail to give the colour reaction, and on the other hand, inactive substances which produce the coloration in question.

The colour test was applied by us mostly to various chemical preparations which were tested out biologically in connection with an investigation in which one of us (S. S. Z.) is at present engaged. We soon became aware that although with the majority of the active substances a coloration of varying intensity could be obtained, there was no quantitative relationship between the two tests, and finally, as already mentioned, that it was possible to obtain a biologically active substance giving no coloration with the reagent.

Decitrated lemon juice was prepared in the usual way, namely, by precipitating the acids and other dissolved substances of the juice with excess of calcium carbonate and absolute alcohol, filtering, removing the alcohol in a vacuum at 40–50° in an atmosphere of carbon dioxide and bringing the solution up to the original volume. A daily dose of 1.5 cc. of such a preparation will keep a guinea-pig on a scorbutic diet alive for at least two months. This solution gave a very intense colour reaction. When it was adsorbed with “norit” (5 g. of “norit” to 100 cc. of solution) in a vacuum for 15 minutes (the utmost care having been taken that the adsorbent was introduced in a vacuum after all the dissolved air of the solution had been previously removed by exhaustion) the solution lost some of its antiscorbutic potency, since a daily dose of 1.5 cc. failed to protect a guinea-pig from scurvy; a daily dose of 3 cc., however, prolonged the onset of scurvy, whilst 5 cc. protected the animals for about 52 days, when the experiment had to be discontinued. Only slight scorbutic signs were observed at the autopsy. In spite of the diminished antiscorbutic potency this preparation was, therefore, definitely active, yet no coloration could be obtained with the modified Folin reagent as prepared by Bezssonoff. The above preparation was freshly made up every day before being administered to the animals. During the dosing periods it was tested colorimetrically on eighteen occasions before the animals were dosed and only on three occasions an extremely faint reaction was obtained. On the remaining days the preparation gave negative tests. The tests were carried out, as was done by Bezssonoff, with 1 cc. of the solution diluted with 1 cc. of distilled water to which five drops of the reagent were added. When no coloration was obtained within 30 minutes, undiluted solutions with varying quantities of the reagent were tried for confirmation purposes.

Further experiments showed the possibility of obtaining a positive colour reaction with antiscorbutically inactive substances, viz. yeast and yeast-extract. Brewer's top-fermentation yeast was washed with water and centrifuged. The washed yeast was then dried for 60 hours at 37°. The dried yeast was digested for 3 hours with about ten times its weight of water and centrifuged. On addition of six drops of the reagent to about 2 cc. of the extract a blue coloration was produced immediately with the formation of a precipitate. This precipitate was centrifuged off after standing with the reagent for 10 minutes and a clear blue supernatant solution was obtained.

Similarly, the yeast preparation “marmite” when treated with a few drops of the reagent is precipitated at first and on a further addition of the reagent an olive-green coloration is produced. When the blue coloration obtained with an active substance is compensated in a comparator with untreated marmite the same olive-green coloration is observed.

The above experiments are sufficient to show that the colorimetric test for the antiscorbutic factor suggested by Bezssonoff is not reliable enough to be of any use in the detection of the vitamin. One need only consider the investigation referred to above to see how wrong conclusions might have been arrived

at by depending on the colorimetric reaction alone. The fact that a great number of antiscorbutic substances also give the test is no proof of the specificity of the reagent for the detection of the vitamin.

It is of interest, however, to record that the substance which gives the blue reaction with the modified Folin reagent employed by Bezssonoff is like the vitamin destroyed by oxidation.

Decitrated lemon juice heated at 100° in an atmosphere of carbon dioxide gives the reaction, whilst a similar juice heated in air does not do so. Also a juice made *N*/20 alkaline and allowed to stand exposed to the air for 2 hours at room temperature no longer gives the coloration. This behaviour is similar to that observed [Zilva, 1922, 1923] in the inactivation of the antiscorbutic factor in decitrated lemon juice under the above conditions, which was shown to be due to oxidation.

Thanks are due to the Medical Research Council for a whole time grant made to one of us. (S. S. Z.)

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CVIII. THE ABSENCE OF EFFECT OF INSULIN ON THE HEAT PRODUCTION IN ISOLATED FROG'S MUSCLE.

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(Received October 9th, 1923.)

WE have carried out a few experiments on the effects of insulin on the heat production of muscle, and although the results so far are merely of a negative character, it seemed to us to be worth while to put them on record since they help at least to restrict the possible methods by which insulin may act upon the system.

For the first four experiments, the insulin used was of the "crude" type, very kindly supplied to us by Messrs Smith and Winter and prepared according to Dudley's method [1923] at the Biochemical Laboratory, Cambridge. Its strength may be taken as being about 5 mg. to the "unit." For the last two experiments, the insulin used was of the "A-B" Brand supplied by the British Drug Co. Its strength is 0.1 cc. to the "unit."

The muscles employed were the sartorius of *Rana temp.*, these being mounted on a thermopile, and the heat production after stimulation being observed by recording photographically the curves of deflection of the galvanometer in series with the thermopile, as fully explained elsewhere [1920, 1922]. The temperature was 14° to 15°. In the present series of experiments, the better to maintain their condition, the muscles were always kept in a salt solution in the thermopile chamber. In this case the actual heat production cannot be calculated from the observed curves, since there is no possibility of making control observations under the same conditions. It is possible, however, by first taking the curves when the muscle is in ordinary Ringer's solution, and then taking another set of curves after insulin has been added, to make a fairly correct estimate of any *change* in the heat production which may be produced by the insulin.

The strength of the insulin in the solution around the muscle was varied between wide limits, but even the weakest solution used would be considered strong if injected into a small animal to make the same concentration in its body.

The chamber containing the muscle was 40 cc. in volume, of which the muscle occupied 0.25 cc.

In Exp. 1 there was 1 mg. of freshly prepared crude insulin in the chamber. In Exp. 2 there were 4 mg. of crude insulin, which had been put into solution on the previous day. In the interval the preparation had been kept on ice.

¹ Working on behalf of the Medical Research Council.

In Exp. 3 there were 4 mg. of freshly prepared crude insulin. In Exp. 4 there were 16 mg. of crude insulin which had been put into solution on the previous day and kept in the interval on ice. In Exp. 5 there was 1 cc. and in Exp. 6 there was 0.5 cc. of insulin "A-B" Brand. The first readings after adding the insulin were taken about an hour after the insulin had been given, and the readings were continued on muscles continuously subjected to the insulin solution for several hours.

RESULTS.

A. *Initial Heat.* Even with the strongest solution of insulin employed the condition of the muscle was apparently unaffected, the initial heat production being practically the same after the muscle had been for five hours in the solution with insulin as it was in the Ringer's solution before insulin was added. Presumably, therefore, the initial breakdown of glycogen or of lactic acidogen into lactic acid is completely unaffected by the presence of insulin.

B. *Recovery Heat.* On comparison of the long continued galvanometer curve for about ten minutes, to see if there were any change in the recovery heat production, a distinct difference was at first detected, showing apparently after insulin had been given that the recovery heat in its earlier stages was less and its later stages more than before insulin. Control experiments, however, were subsequently made on two or three occasions in which long-continued curves of galvanometer deflection were compared (a) for a muscle before, and (b) for a muscle after being kept in Ringer's solution for several hours. In every case a difference was found between the late and the early curves exactly as between those before and after insulin. This change is presumably due to small alterations in reaction or in the physico-chemical condition of the muscle produced by keeping it in Ringer's solution. There seems to be little doubt, therefore, that even a very strong solution of insulin surrounding an isolated frog's muscle has no noticeable effect upon the oxidative breakdowns involved in the recovery process.

In order to ascertain whether the insulin used has any effect on the frog, six injection experiments with insulin "A-B" Brand were carried out. In two of them 0.25 cc. and in four 0.5 cc. were injected into the dorsal lymph sac. About 24 hours after the injection the frogs were found very weak, relaxed, passive and pale, and in two cases were found dead within 48 hours. By the injection of 0.5 cc. of 4 % glucose solution, the suffering frogs seemed to recover temporarily. By repeated injections of glucose, two frogs recovered completely, while one lived for five days without showing any sign of convulsions. Only one frog (which had been injected with 0.25 cc.) showed proper convulsions. After six days this frog fell into strong convulsions (just like the strychnine convulsions) which ceased promptly on the administration of glucose. On the next day, the convulsions were again produced by touching the frog and were again stopped by glucose. Two days after the frog was found dead.

SUMMARY.

Experiments have been performed on the sartorius muscle of *Rana temp.* comparing (a) the magnitude of the initial anaerobic heat production, and (b) the magnitude and course of the oxidative recovery heat production before and after subjection to a high concentration of insulin. No change whatever has been found in either case. The injection of insulin, on the other hand, into the intact frog was shown to produce a well-defined effect.

It is clear, therefore, that the action of insulin does not reside in any effect on the muscle, either on its sudden initial anaerobic breakdowns or its slower oxidative processes of recovery.

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CIX. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM* *COLPODA*. PART II.

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(Received October 8th, 1923.)

IN a previous paper [Cutler and Crump, 1923] an account of experiments on mass cultures of *Colpidium colpoda* was given; the present communication deals with similar experiments carried out on cultures containing one or more cells isolated into one cubic centimetre or less of culture solution. The objects of these experiments have been to discover whether the results obtained from cultures containing few organisms in small quantities of fluid are comparable with those in which numerous animals are inoculated into comparatively large amounts of liquid, 10 cc. or more. Also there have appeared a series of papers by Robertson [1921, 1922], describing new and interesting phenomena—allelocatalysis, etc.—in cultures of a ciliate *Enchelys farcinem*. These seemed to be of so fundamental a character that it was felt desirable to test whether they obtained with other species of ciliates.

METHODS.

The medium used has been the synthetic one in which the experiments on mass cultures were conducted. The smaller cultures (*i.e.* less than 0.09 cc. in volume) were put up as follows in unruled counting chambers 0.1 mm. deep: the individual, or individuals, are transferred, by a capillary tube from the parent culture, with about 0.01 mm.³ of liquid, to the chamber. It has not, however, been possible to keep this quantity rigidly constant, owing to the small volume of the fluid used; the chamber is then covered with a thick cover slip, on which there is already a drop of new medium, freshly inoculated with *Sarcina* to provide an adequate food supply, and the two drops are allowed to mix. The final size of the drop may vary from 0.37 to 9.8 mm.³, and the degree of dilution varies between 1 in 37 and 1 in 980; these dilution figures, however, are only an approximation owing to the difficulty of measuring the original minute drops containing the organisms with accuracy, and, for the same reason, the size of the drop is not of necessity an index of the degree of dilution. Before the cover slip is put on the chamber a ring of small drops of sterile medium are placed in the groove around the raised central part to discourage

evaporation from the culture itself. The drop size in any culture is measured by projecting an image of the chamber on to squared paper, where the outline of the drop is drawn; as the magnification and the depth of the liquid (0.1 mm.) are both known, by counting the squares enclosed by this outline, the volume can be calculated. Each culture has been measured in this way every day so that changes in volume due to evaporation or condensation are detected. The numbers of animals present are counted by projecting the image of the chamber on to a screen, using a 48 mm. lens, and adjusting the eye-piece and distance of the screen so that the final magnification is from 50 to 60 diameters. With practice numbers of animals up to 100 can be counted with a sufficient degree of accuracy.

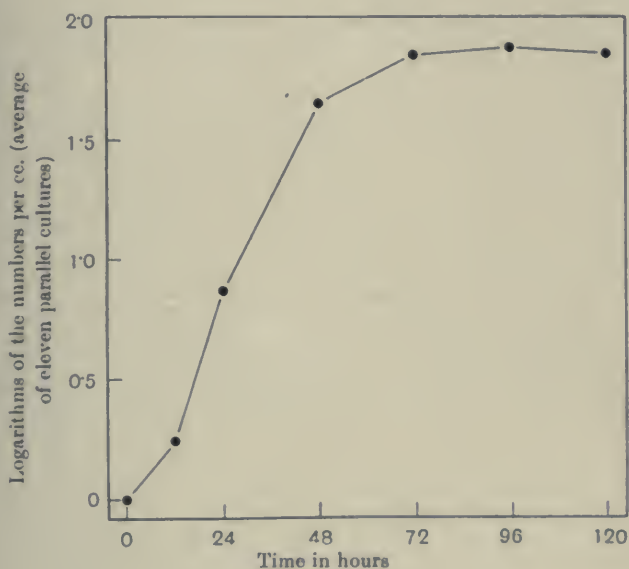


Fig. 1.

RESULTS.

The curves obtained by plotting the numbers of animals in these single cell cultures against the time are similar to those derived from mass cultures, at least until the maximum numbers are reached; after this point, although some of the cultures show the same oscillations that occur in mass cultures, a certain number die out completely in 24 hours. Fig. 1 shows the curve obtained by averaging the numbers of animals every day in eleven parallel cultures (I series, 1 day parent), each starting from a single cell, and plotting the logarithms of these numbers against the age in hours (cf. Fig. 2 in previous paper). It will be seen that the sequence of events in these single animal cultures is in the main similar to that in mass cultures.

Allelocatalysis.

To test the allelocatalytic effect described by Robertson [1921, 1922] a number of experiments were made in which one, two, three or four animals were inoculated into drops of various sizes. The cultures were examined, usually after 12 hours' incubation, and then daily until discontinued. In Tables I and II are given the rates of reproduction arranged in order according to the size of the drop or to the equivalent number of animals inoculated per cc. of liquid. It will be seen that we have been able to find no evidence of the effect obtained by Robertson; for, according to his results, the reproductive rate should increase as the number of animals inoculated increases (Table I); and, the greater the initial concentration per cc., the greater the rate of reproduction should be (Table II). Nor does the size of the drop, within the limits of the experiment, appear to have any appreciable effect, though there is an indication that the animals in the larger drops (giving a low initial concentration) are capable of reproducing a little more rapidly than in the converse case. This would also appear to be in direct opposition to Robertson's theory of the necessity for the animal to develop a definite concentration of "X substance" before division can take place. It should be noted, however, that Robertson [1921] worked with a much larger drop than we have found possible (0.08 cc.) and when only one animal was inoculated the concentration would be only 12.5 per cc. This would materially affect the experiment, but, as *Enchelys farcinem*, the organism with which he worked, measures 50–70 μ long by 35–50 μ broad, while our variety of *Colpidium colpoda* is only about 45–55 $\mu \times$ 10–20 μ^1 , the amount of protoplasm in a drop of the size that Robertson used and the amount in our large drops would not be markedly different. If, then, the quantity of "X substance" produced is at all proportional to the amount of protoplasm producing it, Robertson's results and ours are at variance. It is realised, however, that such an argument may be erroneous, but we would emphasize the fact that the "allelocatalytic effect" does not obtain with *Colpidium colpoda* isolated in drops of the varying sizes we have used. In his last paper, Robertson [1922] states that with *E. farcinem* "single individuals isolated into volumes exceeding 1 cc. very rarely survive and failures to reproduce on the part of individuals isolated into cultures exceeding 0.1 cc. in volume are not at all infrequent." This, as the author points out, is a common experience with micro-biologists.

Assuming that it is due to the inability of the organism, *E. farcinem*, to produce a sufficient concentration of the X substance to induce reproduction, a smaller animal such as *C. colpoda* ought to be incapable of division when isolated into 1 cc. of culture fluid. Experiments were therefore made to test this hypothesis. Here single animals were isolated into 0.25 cc. (4 per cc.), into 0.33 cc. (3 per cc.), into 0.5 cc. (2 per cc.) and into 1 cc. (1 per cc.); two animals were also isolated into 0.25 cc. (8 per cc.) and into 0.5 cc. (4 per cc.) of

¹ The sizes of the animals given in the footnote on p. 179 of our previous paper [Cutler and Crump, 1923] should be 30 $\mu \times$ 10.5 μ and 38.9 $\mu \times$ 21.6 μ respectively.

Table I. *Table showing the reproductive rates of 1, 2, 3 and 4 animals during the first 24 hours after inoculation in drops of varying sizes.*

Size of drop in cubic mm.	1 animal inoculated	Av.	2 animals inoculated	Av.	3 animals inoculated	Av.	4 animals inoculated	Av.
0-0.5	—	—	2.4, 2.7	2.55	—	—	—	—
0.5-1	2.0, 0.0, 1.0, 1.0, 2.0 2.0, 3.5	1.64	2.3, 0.7, 1.8, 2.5, 1.0 1.8, 2.1, 2.5, 3.2	1.99	1.6, 1.7, 3.2, 2.1, 2.4	2.20	1.7, 1.7, 3.3, 1.3	2.00
1-1.5	2.0, 2.0, 3.0, 2.0, 1.0 1.0, 2.0, 0.0, 3.9, 3.8 3.3, 2.0, 2.0	2.15	3.6, 2.3, 3.6, 3.9, 2.3 3.7, 2.5, 2.5	3.05	1.9, 2.0, 2.9, 2.7, 0.7 2.5, 3.8, 3.6	2.51	1.8, 3.3, 3.1	2.71
1.5-2	3.0, 4.0, 1.0, 2.0, 3.1 1.5, 1.0	2.23	1.0, 3.4, 2.5, 3.6	2.62	3.0, 2.8, 1.0, 2.9, 3.7 2.5, 3.4, 3.6, 2.1, 1.2 1.6, 2.6	2.53	2.6, 3.6, 2.0, 2.5	2.67
2-2.5	2.0, 3.9, 1.0, 1.0	1.97	3.8, 2.7, 2.0, 1.5, 1.3	2.26	2.2, 2.3, 3.4, 1.6, 1.9 1.6	2.16	3.3, 2.0, 2.2	2.83
2.5-3	3.0, 3.0	3.00	1.0, 3.4, 2.3, 1.3	2.00	1.4	1.40	1.5	1.50
3-3.5	3.0	3.00	—	—	2.9, 2.7, 4.1, 2.0	2.92	2.7, 3.5, 3.5, 1.3	2.75
3.5-4	1.5, 4.0, 4.4, 4.6, 3.8 2.0	3.38	—	—	2.3, 1.7	2.00	—	—
4-4.5	2.0, 3.0, 2.0, 3.0	2.50	—	—	4.1	4.10	1.7, 2.4	2.05
4.5-5	3.9, 1.0	2.45	3.0	3.00	—	—	2.8, 2.1	2.45
5-5.5	2.0, 2.8	2.40	4.5	4.50	1.0	1.00	—	—
5.5-6	2.0, 3.3, 3.1	2.80	—	—	1.4	1.40	—	—
6-6.5	—	—	2.1, 3.8	2.95	—	—	—	—
6.5-7	3.7, 1.5	2.60	—	—	—	—	—	—
7-7.5	—	—	4.3, 1.0	2.65	—	—	—	—
7.5-8	—	—	—	—	—	—	3.3	3.39
8-8.5	3.4	3.40	3.0	3.00	—	—	—	—
8.5-9	—	—	—	—	—	—	3.3	3.30
Average for drops of all sizes								
	2.4		2.5		2.4		2.5	

Table II. *Table showing the reproductive rates during the first 24 hours in cultures whose initial concentrations vary from 100 to 8000 per cc.*

Number of animals per cc. inoculated	Reproductive rate for the first 24 hours	Av. rate of reproduction for the first 24 hours
100-200	2.0, 3.9, 3.3, 2.0, 2.5, 3.1, 3.4, 2.5, 3.7	2.96
200-300	2.5, 1.0, 1.0, 2.0, 3.9, 3.0, 1.0, 3.0, 3.0, 3.8, 2.0, 4.0, 4.0, 4.2, 4.5, 1.0, 2.0	2.70
300-400	3.9, 3.0, 3.0, 2.1, 3.8, 4.5	3.39
400-500	2.0, 2.0, 1.0, 3.3, 3.0, 1.0	2.05
500-600	3.0, 0.0, 4.0, 2.5, 1.0, 3.3, 2.0, 1.0, 1.0, 1.4	1.92
600-700	2.0, 1.8, 3.9, 3.1	2.70
700-800	2.0, 3.0, 1.0, 1.5, 1.0, 2.3, 1.0, 2.0, 3.4, 4.1, 3.3, 2.3	2.23
800-900	1.0, 2.0, 3.7, 2.9, 2.8, 2.7, 2.0, 2.4, 1.7, 2.0, 2.0, 2.5, 1.7	2.23
900-1000	1.0, 0.0, 1.0, 2.0, 1.0, 1.0, 3.8, 2.7, 1.7, 4.1, 2.0	1.84
1000-1100	3.5	3.50
1100-1200	2.5, 2.7, 1.4	2.20
1200-1300	3.3, 2.2, 3.6, 3.5, 3.6, 3.6, 3.4, 1.8, 1.5, 1.3	2.78
1300-1400	1.0, 2.0, 1.7, 1.6	1.57
1400-1500	2.0, 2.3	2.15
1500-1600	2.0, 1.0, 3.7, 2.3, 3.6, 2.5	2.51
1600-1700	1.0, 3.5, 2.5, 3.7, 3.3, 2.6	2.76
1700-1800	2.9, 2.1, 1.6	1.40
1800-1900	2.9, 2.1, 1.6, 1.0, 3.0	2.12
1900-2000	0.0, 2.5, 3.6, 2.0, 3.3, 3.8, 3.1, 2.1, 1.2, 2.5	2.41
.....		
2000-3000	2.0, 2.6, 3.5, 1.0, 0.7, 2.5, 1.8, 1.9, 3.3, 3.1, 0.7, 2.1	2.10
3000-4000	2.4, 0.5, 2.3, 1.8, 1.0, 2.5, 3.1, 1.8, 2.1	1.94
4000-5000	3.0, 1.2, 2.7, 1.3	2.05
5000-6000	0.0, 2.4, 1.6, 1.6, 1.7	1.46
6000-7000	1.0	1.00
7000-8000	3.2	3.20

liquid. The medium used in these experiments was one in which 0.024 % of ammonium phosphate and 0.016 % of glycerol were substituted for the sodium phosphate, ammonium lactate and glucose of the medium used in the other experiments. Where only one animal is present any growth must be due to that animal, but where two are introduced there is always the possibility that one may have died. Attempts were made to find the animals immediately after inoculation, and also to count them by direct examination of a projected image of the culture tube; this can only be very unsatisfactory even in 0.25 cc. cultures, though by using small tubes (2×0.3 inches), and immersing them in water to lessen refraction, a satisfactory image can be obtained and a rough approximation to the number of animals made. When the numbers rise to 400 per cc. or more, counts are made in a counting chamber.

The following table shows the figures obtained.

Table III.

Initial no. per cc.	No. of parallels	Average reproductive rate
1	5	14.94
2	5	13.80
3	5	13.60
4	7	12.72
8	2	11.05

These results show that the organism is capable of vigorous reproduction even when isolated into relatively large volumes of liquid¹.

Acceleration due to X substance.

The experiments detailed above demonstrate that the action of the growth promoting substance X is not so simple as might be thought by a perusal of Robertson's communications; but the work of this author and of others leaves little doubt but that some kind of accelerative agent is produced during the growth of organisms in culture fluid.

Since the publication of Wildiers' paper [1901] on the growth of yeast cells, where it is shown that the yeast does not develop its optimum reproductive capacity in the absence of an unknown substance, termed by Wildiers "bios," many investigations have been made to determine the nature of the accelerative agent. This work need not be detailed here as already many summaries have been made. It may be recalled, however, that Cantani [1901] reports that the growth of *Bacillus influenzae* is enriched by the presence of *C. diphtheriae*, *M. gonorrhoea* and certain staphylococci; a result also obtained by the use of dead bacteria as the stimulating agent. Such observations have been confirmed and extended by Neisser [1903] and Thjötta [1921, 1, 2]. Very few observations have been made on organisms other than bacteria, though Haberlandt [1913, 1920] adduces evidence that the plant cell produces a substance that is capable of inducing rapid cell division.

In view of the importance of these conclusions we have performed similar

¹ That single bacteria are also able to grow when inoculated into relatively large volumes of medium has been shown by Barber [1908], Churchman and Kahn [1921], and others.

experiments on *Colpidium colpoda*. The results, given in Table IV, show that crushed *Sarcina* or crushed *Colpidia* have the power of accelerating cell division; though the action does not seem to occur during the first 24 hours of incubation. This is contrary to the findings of Robertson, and is difficult to understand on the analogy of enzyme action.

Also the possibility has not been excluded that the increased reproduction is due to an increased food supply afforded by the crushed fragments of protozoa or bacteria. Such a possibility, however, obtains also in Robertson's experiments.

It is our intention considerably to extend these experiments, but in view of the interest of the problem it seems worth while to record the few observations already made.

Table IV. *Showing the accelerative effect due to the presence of crushed Sarcina and crushed Colpidium.*

Initial concentration			Reproductive rate at the end of 24 hours' growth.			
	Control	Av.	Crushed Colpidium	Av.	Crushed Sarcina	Av.
100- 500	1.0	1.00	—	—	1.0, 1.0	1.00
500-1000	1.0	1.0, 0.0, 1.5, 1.0, 0.0	0.75	1.0, 2.0, 1.5, 1.0, 0.0, 1.7	1.20	0.1, 0.0
1000-1500	1.0	1.00	1.4, 1.0, 1.0	1.13	—	—
1500-2000	0.0	0.00	0.0	0.00	—	—
2000-2500	1.0	1.00	0.1	0.10	—	—
2500-3000	0.5	0.50	1.4	1.40	—	—
.....						
5000-5500	0.0	0.00	—	—	—	—
6000-6500	1.0	1.00	—	—	—	—
8000-8500	—	—	1.0	1.00	—	—
Average:	0.69		1.00		0.52	
Reproductive rate after 48 hours' growth.						
100- 500	2.0	2.00	—	—	4.8, 3.9	4.35
500-1000	1.0, 2.0, 2.0, 2.9, 2.0, 1.0	1.81	3.8, 4.5, 4.2, 1.8, 2.0, 3.3	3.26	3.7, 4.6	4.15
1000-1500	1.0	1.00	4.3, 1.0, 2.9	2.73	—	—
1500-2000	0.0	0.00	0.0	0.00	—	—
2000-2500	1.5	1.50	2.8	2.80	—	—
2500-3000	0.7	0.70	2.5	2.50	—	—
.....						
5000-5500	0.0	0.00	—	—	—	—
6000-6500	1.4	1.40	—	—	—	—
8000-8500	—	—	3.1	3.10	—	—
Average:	1.34		2.78		4.25	

In connection with the accelerative effect of extracts of *Colpidia* and *Sarcina* it is interesting to note the action produced by the inoculation of fluid from a culture which had grown well into one which had shown a low rate of reproduction. The organism on which this test was made was a flagellate, *Oicomonas termo*. On May 15th, 1922, there was inoculated into 20 cc. of ammonium phosphate + saccharose medium sufficient *Oicomonas* to give a concentration of 70,238 per cc. The culture (Z2) during the first 24 hours decreased in numbers to 51,500 per cc., but at the end of 72 hours had increased to 2,575,000 per cc.; thus showing a reproductive rate of 5.6. Concurrently with this another culture, X1, was started in the same amount of culture fluid but with a concentration of only 17,559 per cc. During the first few hours of incubation the numbers fell to 7000 per cc., and at the end of 72 hours had

reached 13,000, showing a reproductive rate of only 0.89. At this juncture, therefore, a portion of Z2 was centrifuged to eliminate the organisms, and 1 cc. of the supernatant fluid was added to X1a, a culture formed by dividing X1 into two equal 10 cc. portions.

Hours:	0	4	24	48	96	120	144
X 1	13,000	9,500	2,000	2,000	under 1000	under 200	under 200
X 1a	13,000	17,500	31,000	20,000 + cysts	21,000	15,000	2,000

From the above table it is seen that, while the control culture X1 steadily decreased in numbers, the treated X1a culture, after 4 hours, exhibited the effect of the treatment, and after 24 hours had had a reproductive rate of 1.25.

The experiment is also of interest in demonstrating that the decrease in numbers in an old culture is not primarily conditioned by the culture fluid becoming toxic: for the numbers of Z2, whose culture fluid was added to form X1a, steadily fell after the beginning of the experiment. This conclusion has been independently arrived at by Robertson from other evidence, and was also put forward by us in our previous paper.

Rate to first maximum.

In our previous paper it was shown that, when the logarithms of the numbers inoculated into a constant volume of medium were plotted against the maximum numbers attained, a reproductive rate varying from five to six divisions was found for 91 % of the cases. This held for inocula of 100 to 700 per cc., for parent cultures from 24 to 48 hours old, and the time required for the divisions varied from 48 to 168 hours.

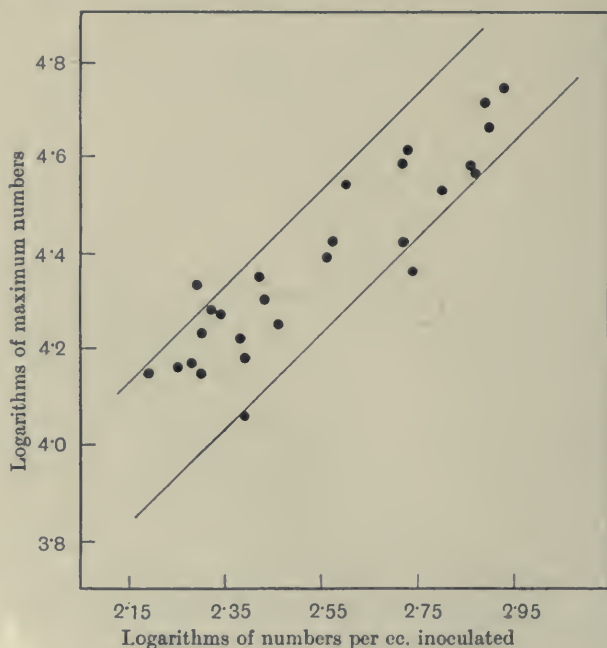


Fig. 2.

In the single cell experiments described above the majority of the cultures were not kept sufficiently long to attain their maximum numbers, but in 29 cases this was done. When the procedure adopted for the mass cultures is used it is found that again the reproductive rates fall within narrow limits, but instead of being from 5 to 6 divisions they are from 5.5 to 6.5 divisions (Fig. 2). This obtains, however, only when the initial concentration is equivalent to numbers lying between 100 and 900 per cc. Within these limits the age of the parent culture may be 24 or 48 hours, and the time taken to reach the maximum has varied from 72 to 168 hours. With concentrations above 900 per cc. the reproductive rates fall rapidly, as is shown in the following table:

Culture	Initial concentration per cc.	Rate	Final concentration per cc.
<i>F</i> 4	906	3.00	8,384
<i>H</i> 10	912	5.46	37,620
<i>G</i> 8	920	5.42	40,334
<i>H</i> 2	981	4.80	39,236
<i>G</i> 2	997	4.08	17,459
<i>G</i> 10	1,560	4.00	23,850
<i>H</i> 19	1,686	4.50	40,894
<i>I</i> 4	1,704	3.93	25,856
<i>H</i> 18	1,720	3.70	23,582
<i>B</i> 17	1,857	3.62	30,784
<i>B</i> 10	2,380	3.70	34,700
<i>D</i> 1	2,475	1.50	8,534
<i>G</i> 12	2,838	1.73	12,507
<i>D</i> 2	3,220	0.80	5,220
<i>E</i> 2	5,292	0.00	5,292
<i>E</i> 6	6,116	1.10	11,830

It was thought possible that the high concentration giving a low reproductive rate might be explained as follows: suppose each organism was capable of dividing 5 times, and, that, at a certain concentration x , autointoxication occurred; then, it might be, that when the initial concentration was high, the critical concentration x was obtained before 5 divisions had occurred. This, however, will not explain the result, since a culture *G*5, which had initially two animals and a concentration of 848 per cc., divided 5.9 times and attained a final concentration of 54,570 per cc. From the above table it will be seen that the final concentrations, in those cultures showing a depressed rate of reproduction, are below that of *G*5; though in the majority of cases if an extra division had taken place the concentration would have been above or near to that found in *G*5. Also with concentrations considerably below 100 per cc. the rate of reproduction does not fall within the limits 5.5-6.5, but is considerably increased as is shown in Table III.

These results indicate that with inocula of this size the animals tend to reach a constant maximum concentration instead of dividing a definite number of times as they seem to do when the inocula lie between 100 and 900 per cc. This tendency to arrive at a constant population is also seen when part of a mass culture is centrifuged to reduce the number of animals, and the supernatant fluid poured off. The reproductive rate of the animals left in this fluid is higher than it is in those that remain in the original culture.

SUMMARY.

The rate of reproduction of *Colpidium colpoda* has been tested in cultures derived from one or more animals isolated into small volumes of fluid. It is shown that in the main such cultures are comparable with mass cultures.

The allelocatalytic effect, described by Robertson, has been tested for and found not to obtain with *Colpidium* when isolated into fluid whose volume varies from 0.5 to 8.5 mm.³. A few experiments are given in support of the contention that the rate of reproduction can be accelerated by the addition of small quantities of crushed bacteria or protozoa.

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CX. A PRELIMINARY NOTE ON THE DESTRUCTION OF VITAMIN B BY AGE.

By GEORGE MARSHALL FINDLAY.

From the Royal College of Physicians' Laboratory, Edinburgh.

(Received October 30th, 1923.)

ALTHOUGH much work has been carried out on the destruction of vitamin B by such agencies as heat and oxidation, comparatively few investigators have examined the question of the loss in the vitamin B content of natural food-stuffs caused by the mere passage of time.

Vedder and Williams [1916] found that unpolished rice stored in a warm moist place for one year retained its power of curing polyneuritis in pigeons. While this paper was in course of preparation there appeared a communication by Jansen [1923] who states that a sample of rice which had been stored as paddy for 100 years was found to have lost, as compared with new rice, little or none of its anti-beriberi vitamin. The question as to whether the anti-beriberi vitamin is identical with the vitamin B, growth-promoting factor is still unsettled. In the present paper a preliminary account is given of investigations on the vitamin B content of seeds stored under constant conditions for a number of years.

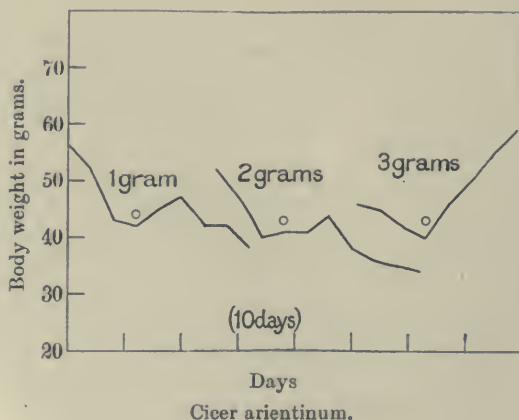
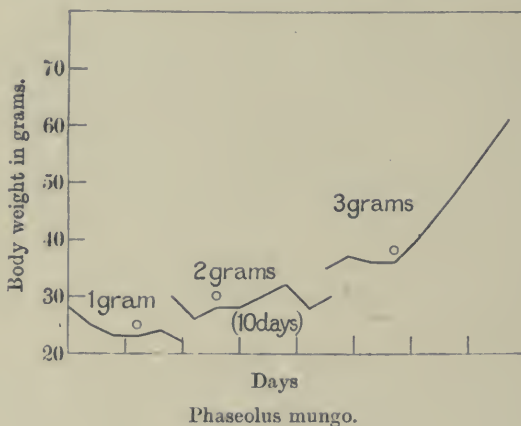
Through the kindness of Professor Watson of the Department of Agriculture of the University of Edinburgh, I was provided with a supply of certain Indian lentils which are known to have arrived in this country in the spring of 1886. They are thus at least 38 years old. During the whole of this period they have been stored in canvas bags in a dry cupboard in the Department of Agriculture. All the seeds had lost the power of germination. The different lentils examined included:

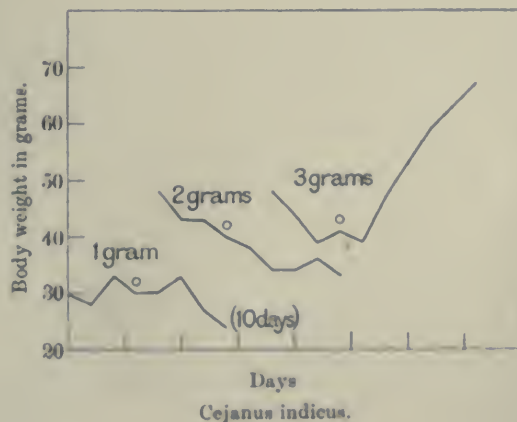
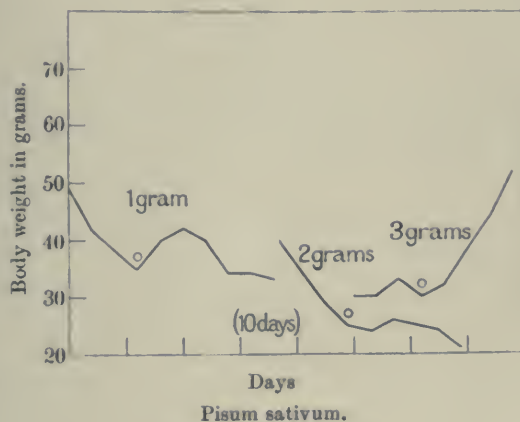
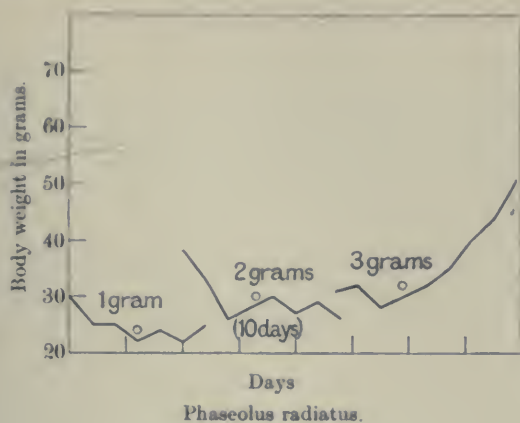
1. *Cicer arietinum* Linn.—“Butt” (yellow variety).
2. *Phaseolus mungo* Linn.—“Sona Moona” (golden yellow in colour).
3. *Cejanus indicus* Spreng.—“Ahar” (yellow-ochre variety).
4. *Phaseolus radiatus* Linn.

Unfortunately, it was not possible to obtain supplies of fresh lentils for comparison with these seeds of 1886, but luckily, their vitamin B content has recently been investigated by Ghose [1922]. Although no data are given by him as to the age of the seeds used, it is to be presumed that they were not more than two or three years old. Ghose found that the addition of 1 g. of any of the above lentils to a standard diet lacking vitamin B was sufficient to promote adequate growth in rats. No observations, however, were made as to whether 1 g. was the minimal amount necessary to ensure growth.

In order to compare my results with those obtained by Ghose, the technique adopted was closely similar, except that all faeces were removed from the cages twice daily, since by devouring their own faeces it is possible that rats may obtain small supplies of vitamin B. The rats used were slightly lighter than those employed by Ghose—the average weight at the beginning of the experiment being about 40 g. The results obtained by the addition of 1, 2 and 3 g. of lentils to the standard diet lacking vitamin B are shown in the charts, where each curve represents the average weight of four rats. It will be seen that growth occurred after the addition of 3 g., but not after the addition of 1 or 2 g.

Experiments were also carried out with *Pisum sativum* which had been stored for 38 years. Here, again, the addition of 3 g. to the standard diet gave adequate growth, while no growth was obtained by the addition of 1 or 2 g. of the pea. The power of germination had been entirely lost.





CONCLUSIONS AND SUMMARY.

Samples of Indian lentils and peas kept for 38 years still contain appreciable quantities of vitamin B. In comparison with the observations of Ghose, however, they appear to have lost a small amount of their vitamin B content, even if allowances are made for the slight differences in technique previously described.

Seeds which have lost the power of germination have not necessarily lost all their vitamin B content.

The whole question of the destruction of vitamin B with age is obviously one which requires further and fuller investigation, more especially in regard to the effects of different climatic conditions, *e.g.* heat and moisture, on the rate of destruction.

I desire to express my thanks to Professor Watson and Dr Smith for their kindness in supplying me with seeds, as also to Lieut.-Col. McKendrick for his continued interest in this research.

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CXI. THE ABSORPTION SPECTRA OF SOME INDOLE DERIVATIVES.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(Received August 8th, 1923.)

IN the course of an investigation on the metabolism of tryptophan and some of its derivatives, it was thought advisable to study the absorption spectra of several of these compounds.

Apparatus.

The apparatus used for the measurement of these absorption spectra was a quartz ultra-violet spectra photometer made by Adam Hilger, Ltd. It consisted of a quartz ultra-violet spectragraph with an electrode holder, a 1/4 kilowatt transformer, a suitable condenser and a rotating sector photometer. The spectragraph took a plate 10×4 inches and also contained a wave length scale mounted inside the spectragraph. By means of this scale the wave length could be photographed on the plates.

For electrodes, nickel rods were found to be the best for this kind of work. The emission spectrum of nickel is nearly as rich in lines as that given by iron and continues as far down as 2200 A.U. whereas the spectrum of iron has relatively few lines below 2500 A.U. Copper gives an emission spectrum which goes as far down as 2100 A.U. but the lines are not nearly as plentiful and it does not give nearly as satisfactory results in the measurement of absorption spectra.

Method.

In the Adam Hilger spectra photometer, simultaneous adjacent pairs of photographs of the spark across the electrodes are taken, one of which is through a cell containing the solution of the material to be measured and the other through a cell containing the solvent. The photograph through the solvent is to be considered the standard with which the other is to be compared. A number of such pairs of photographs are taken and at the finish of each exposure the sector wheel in front of the solvent or standard is closed down, the amount of light allowed to pass through by the sector wheel unto the photographic plate being in a definite ratio to the light that would pass through the solution providing no absorption were taking place. After the plate has been completely exposed, developed, washed and dried, the point of equal blackening is marked on each of the simultaneously taken adjacent pairs of photographs.

The absorption curves are plotted on cross section paper in which one ruling is arithmetic and the other logarithmic. The wave length is plotted on the arithmetic scale and the molecular absorption coefficient on the logarithmic scale. The molecular absorption coefficient is calculated from the following equation.

$$M = \frac{1}{d} \times \frac{\log I_0 - \log I}{C}.$$

The Adam Hilger sector photometer is graduated to read in terms of

$$\log I_0 - \log I.$$

Hence the readings on the sector wheel may be called a and the equation then becomes

$$M = \frac{1}{d} \times \frac{a}{C},$$

where M is the molecular absorption coefficient,
 d is the thickness of the absorbing layer in centimetres,
 $\log I_0$ is the logarithm of the wave-length of the incident light,
 $\log I$ is the logarithm of the wave-length of the transmitted light,
 and C is the molecular concentration.

Experimental.

In all ten different compounds were measured. These ten compounds form four groups.

Group I: Indole, β -indolecarboxylic acid, and β -indole-aldehyde.

The indole was obtained from one of the drug houses. It was carefully purified by boiling with animal charcoal and recrystallising from dilute alcohol.

The β -indolecarboxylic acid was prepared by means of the Grignard reaction [Majina and Kotake, 1922]. It was recrystallised by dissolving in acetone and pouring into water [Ellinger, 1906]. This gave a white crystalline solid melting at 213° .

The β -indole-aldehyde was prepared by oxidising tryptophan with ferric chloride [Hopkins and Cole, 1903; Ellinger, 1906]. It was boiled with animal charcoal and recrystallised from dilute alcohol.

Fig. 1 shows the absorption curves of these compounds.

Group II: β -Indole-ethyl alcohol, β -indolepropionic acid and β -indole-aminopropionic acid or tryptophan.

The β -indole-ethyl alcohol was prepared from tryptophan by the action of yeast fermentation [Felix Ehrlich, 1922]. It was decolorised by boiling with animal charcoal and carefully recrystallised from very dilute alcohol.

The β -indolepropionic acid was prepared from tryptophan by the action of anaerobic bacteria under anaerobic conditions. The bacteria used were a mixture of *B. coli*, *B. chauvei*, *B. sporogenes* and *B. oedemaciens* [Nencki, 1889; Hopkins and Cole, 1903]. The introduction of the four different strains is a modification of the previous methods and will be published in more detail elsewhere. The indolepropionic acid was carefully decolorised with animal charcoal and recrystallised from dilute alcohol.

The tryptophan was prepared by the original method of Hopkins and Cole [1901]. It was very carefully decolorised with animal charcoal and recrystallised from 65 % alcohol.

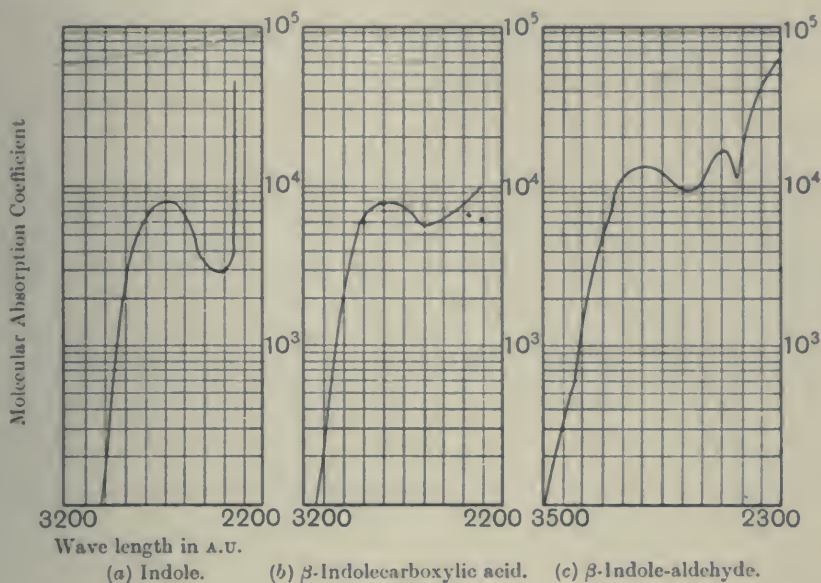


Fig. 1.

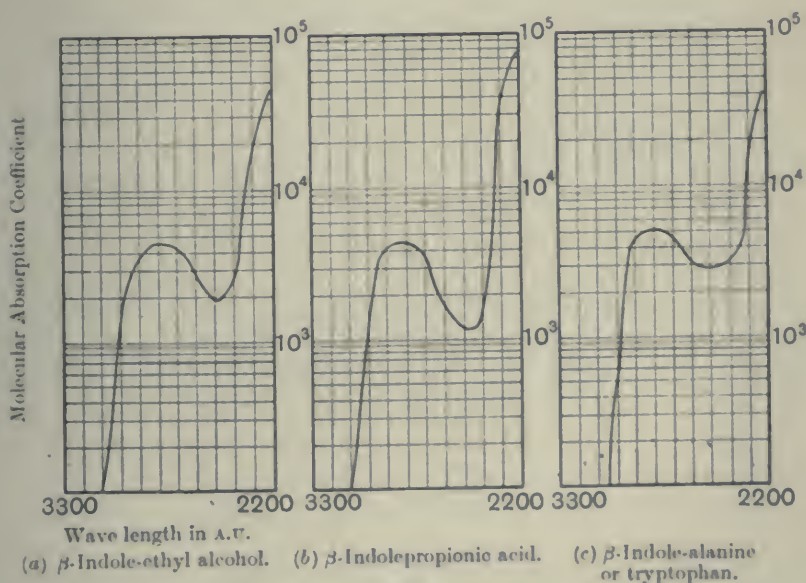
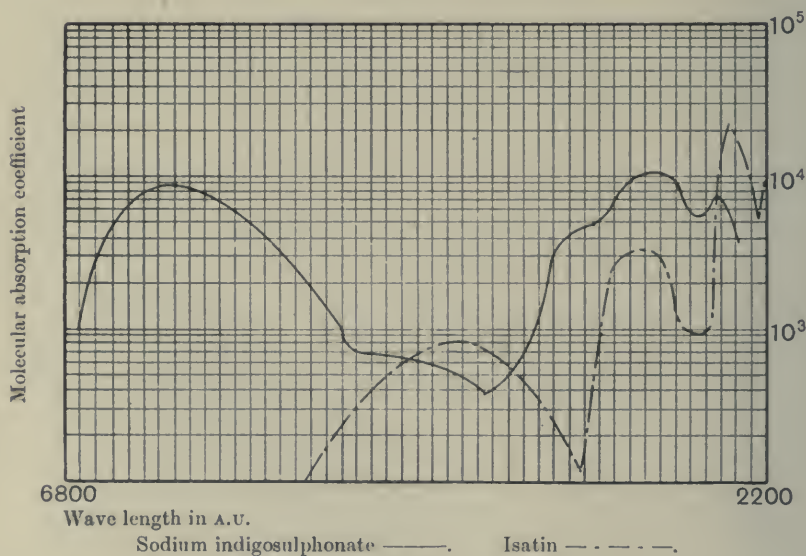
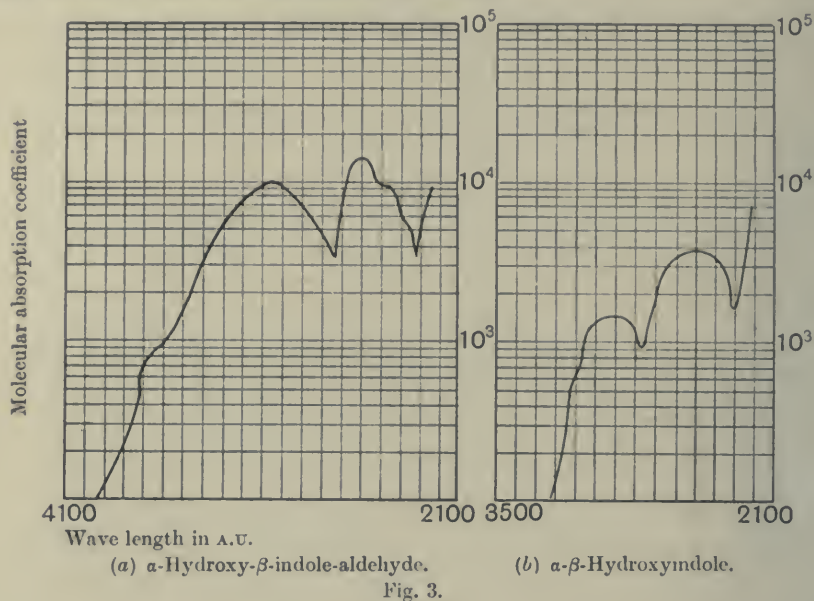


Fig. 2.

Fig. 2 gives the absorption curves of these compounds.
Group III: α - β -Dihydroxyindole and α -hydroxy- β -indole-aldehyde.

The α - β -dihydroxyindole was prepared from isatin by reducing with sodium hydrosulphite [Marschalk, 1912]. It was carefully decolorised with animal charcoal and recrystallised from water.



The α -hydroxy- β -indole-aldehyde was prepared from thio-indigo Scharlach, R. Kalle and Co. [Friedländer and St Kielbasinski, 1912]. It was boiled with animal charcoal and recrystallised from dilute alcohol. Pale yellow crystals were obtained melting at 208° .

Fig. 3 shows the absorption curves of these compounds.

Group IV: Isatin and sodium indigosulphonate.

The isatin was obtained from a drug house and carefully boiled with animal charcoal. It was then recrystallised from dilute alcohol.

The sodium indigosulphonate or indigo carmine was a sample of Kahlbaum's pure chemicals and was used without further purification.

Fig. 4 shows the absorption spectra curves.

Discussion.

In examining the charts of group I, namely indole, β -indolecarboxylic acid and β -indole-aldehyde, one may observe the effect of substituting carboxyl and aldehyde groups in the β -position of the indole ring. Indole has a band with the tip at 2700 A.U. and with a molecular absorption coefficient of about 9000. The effect of substituting a carboxyl group in the β -position is to shift the band about 100 A.U. towards the red end of the spectrum but the intensity of the absorption is not changed. The effect of substituting an aldehyde group in the β -position is different. The band is also shifted towards the red end of the spectrum but the shift is much greater, being about 400 A.U. or four times that due to the carboxyl group. The intensity of the absorption is also increased from a molecular absorption coefficient of 9000 to 15,000.

V. Henri [1912] has shown that the carboxyl group of the aliphatic acids, such as acetic and propionic acid, absorbs the rays in the region of 2200 to 2400 A.U., the intensity increasing and shifting towards the red with increasing molecular weight of the substance. This same type of absorption may be observed in β -indolecarboxylic acid. Henri has also shown that aliphatic aldehydes such as acetaldehyde and propylaldehyde have a characteristic type of absorption curve. These curves rise to a peak around 2600 A.U., fall off again and then rise again in the extreme ultra-violet around 2200 A.U. This same type of curve is found in the lower end of the absorption spectrum of β -indole-aldehyde. Fig. 1 c shows this type of curve.

In examining the charts of group II, which consists of β -indole-ethyl alcohol, β -indolepropionic acid and β -indole-aminopropionic acid, it will be observed that the tips of the bands of all three are at about the same molecular absorption coefficient. It should be noted that these three compounds are substituted compounds of β -ethylindole, the substitution being in the side chain and being firstly a hydroxyl group, secondly a carboxyl group and thirdly an amino and a carboxyl group.

The point to be noted here is that the substitution in this side chain has very little effect in shifting the band towards the red end of the spectrum, although it has been shown that substitution in the ring by a carboxyl group shifts the band 100 A.U. in this sense. The alteration in the absorption follows the course which it would take if the compounds were entirely aliphatic. Henri has shown that the hydroxyl groups of aliphatic alcohols have an absorption band in the region of 2100 to 2200 A.U. This absorption band is

less intense than that given by corresponding aliphatic acid carboxyl groups. It is also further towards the extreme end of the ultra-violet. A similar relation holds between the lower parts of the curves of β -indole-ethyl alcohol and β -indolepropionic acid as can be seen in Fig. 1a and 1b.

In the case of β -indole-aminopropionic acid there is both an acidic and a basic group, giving an opportunity for salt formation. Salt formation has been shown by Henri [1913] to shift the absorption band into the extreme end of the ultra-violet. Salt formation appears to have taken place in this case, since that part of the absorption spectrum corresponding to the amino and carboxyl groups is further in the ultra-violet than the corresponding part for β -indolepropionic acid. The intensity is also less.

The substitution of the side chain in the indole ring acts in a similar manner to the substitution of a methyl group and shifts the absorption band about 50 A.U. towards the red. But the intensity of the absorption is decreased from 9000 to 5000 units.

In examining group III it is seen that the two members are α - and β -disubstituted compounds, the α -substitution being in both cases the same, with the β -substitution in one case a hydroxyl group and in the other case an aldehyde group.

The most striking difference between this group and the previous groups is that instead of having only one main band as in groups I and II there are two well-marked bands, which occupy relatively the same positions to each other. The α - β -dihydroxyindole shows an absorption band in the region of 2200 and 2300 A.U. characteristic of the absorption band in aliphatic compounds having hydroxyl groups. The hydroxyindole-aldehyde also shows this absorption band but, as is to be expected, it is not so pronounced. The aldehyde group substituted in the ring shows the same effect in intensifying the absorption as it does in the case of indole-aldehyde. The shift in the bands towards the red of hydroxyindole-aldehyde compared with dihydroxyindole is about 500 A.U., whereas the shift in the band of indole-aldehyde compared with indole is about 400 A.U. The agreement in the effect of the aldehyde is quite good. The aldehyde absorption band does not show up so distinctly in hydroxyindole-aldehyde as in indole-aldehyde owing no doubt to the fact that the position is already occupied by one of the main bands of the parent substance.

In the case of group IV nothing much can be said owing to the lack of a number of compounds on which to make comparisons. In these two cases the double bond has disappeared from the pyrrole ring and the change in the absorption completely destroys any resemblance to the other three groups. These compounds are not indole compounds but indolin derivatives.

Sincere thanks are due to Professor Hopkins for his kindness in purchasing for the Biochemical Department the very specialised apparatus required for this research.

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CXII. THE ABSORPTION SPECTRA OF SOME AMINO ACIDS.

THE POSSIBLE RING STRUCTURE OF CYSTINE.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(Received August 27th, 1923.)

THE object of this investigation was to find out whether the position of the absorption bands of amino acids bore any relation to the wave length of those ultra-violet rays which have proved most effective in the destruction of micro-organisms and for therapeutic purposes. When an organism is destroyed by the effects of radiation of any particular wave length a certain amount of work must have been done and a certain amount of energy corresponding to this work must have been utilised. The source of this energy is the rays absorbed by the tissues. In view of the fact that amino acids are the elements from which the protein compounds are built up and the fact that protein constitutes a large part of the non-aqueous part of protoplasm it would appear that there is a direct relationship between the absorption spectra of the amino acids and the effect of radiation on organised living matter.

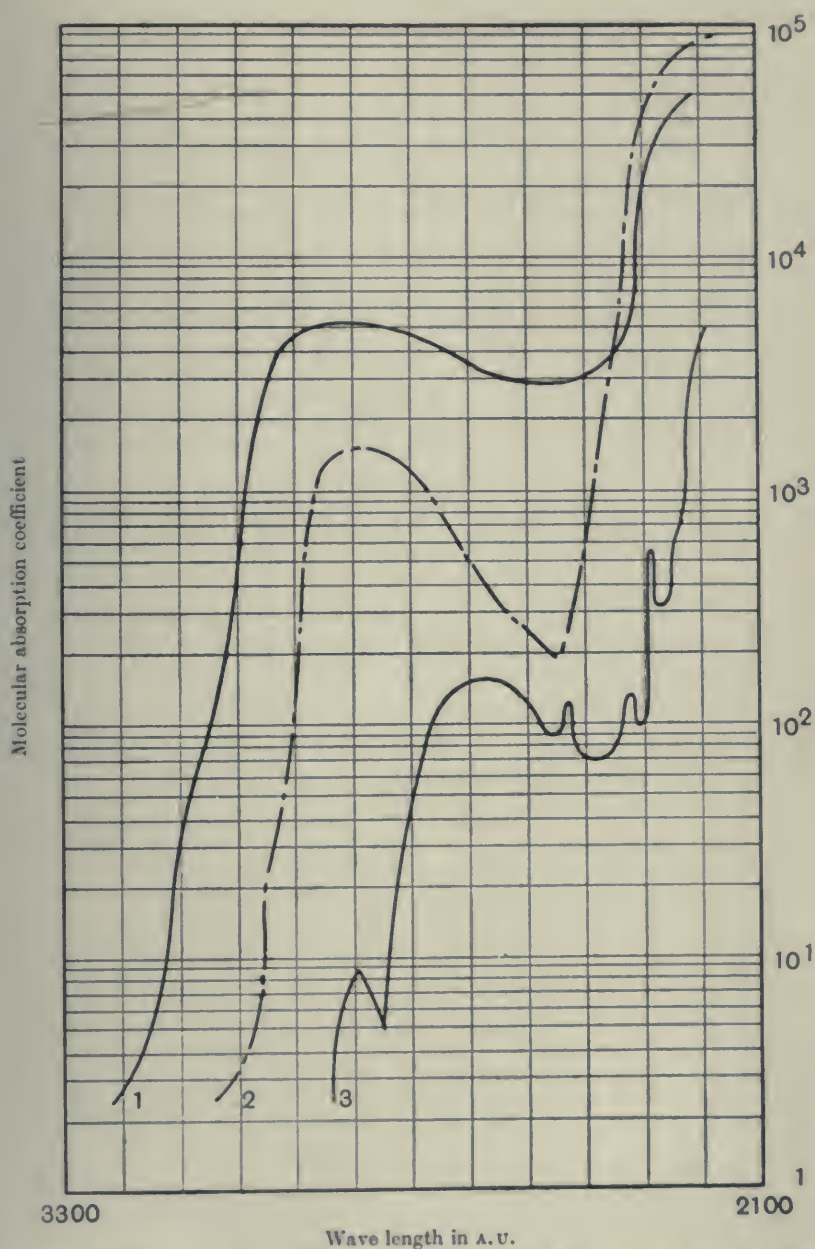
EXPERIMENTAL.

Method and Apparatus.

The method and apparatus employed in the study of the absorption spectra of these amino acids is the same as that employed by the present worker in the study of the absorption spectra of some indole compounds [Ward, 1923].

The absorption spectra of seven amino acids were measured. These were tryptophan, tyrosine, phenylalanine, alanine, histidine, glutamic acid and cystine. Of these seven amino acids, six were prepared in the Cambridge Biochemical laboratory. The remaining one, alanine, was purchased from British Drug Houses.

In making the absorption spectra measurements, solutions of a definite molecular concentration were used in order to obtain results that would have some basis of comparison. The solvent used for dissolving the amino acids was 50 % alcohol. Phenylalanine, tyrosine, histidine, glutamic acid and cystine were used in the form of the hydrochloride. Tryptophan and alanine were used in the form of the free acid. $M/100$ solutions of tryptophan, phenylalanine, tyrosine, and histidine were used as the strongest concentration and dilutions made from these solutions. Alanine and glutamic acid were used in $M/10$ concentration. Cystine was used in $M/20$ molar concentration and treated as if it were $M/10$ in plotting the results in order to obtain comparable results.



(1) Tryptophan. (2) Tyrosine hydrochloride. (3) Phenylalanine hydrochloride.

Fig. 1

The absorption spectra curves are plotted on Figs. 1 and 2. Fig. 1 gives the absorption spectra curves of tryptophan, tyrosine and phenylalanine, Fig. 2*b* those of histidine and glutamic acid and Fig. 2*a* those of cystine and alanine.

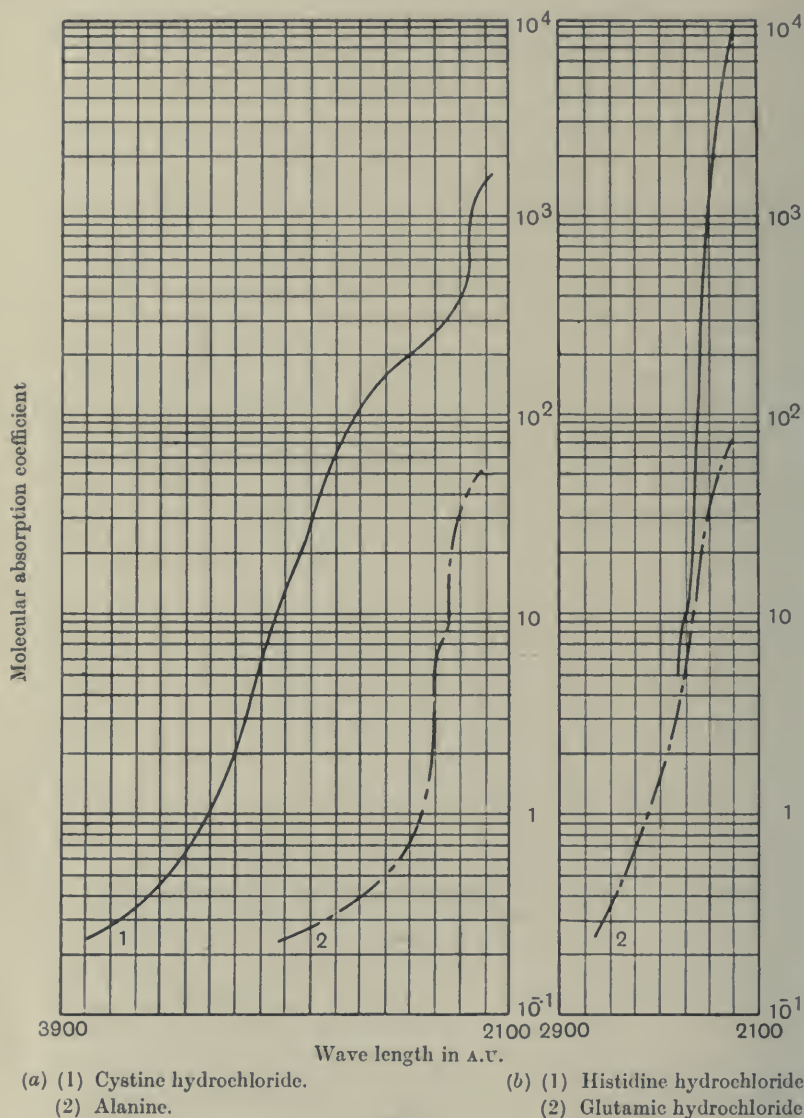


Fig. 2.

Discussion.

Of the seven amino acids examined, the three acids containing the benzene ring—phenylalanine, tyrosine and tryptophan—give marked absorption bands. The others give only general absorption. Cystine gives a general absorption

with an intensity as great as that given by phenylalanine in low concentrations, and in high concentrations the absorption band extends to the edge of the visible spectrum. Kober [1915] has measured the edges of the bands of a number of amino acids without determining the complete absorption curve. From these results, it appears probable that the absorption spectra of the remaining amino acids have curves similar to those of glutamic acid and alanine.

The solar light contains ultra-violet rays that extend down to the wave length of about 3000 A.U. Cystine is the only amino acid apparently that has any marked absorption in the region of the solar ultra-violet light. It would appear suggestive that the presence of cystine in hair and wool where the concentration approaches that of about 10 %, is of physiological importance in the protection of the organism against the harmful effects of prolonged exposure to sunlight. The curious custom of the Arabs in wearing heavy but loosely fitting woollen clothing has apparently a strong justification in the protective effect of the cystine present in the wool.

Cystine appears to be present in high concentration only in the scleroproteins of hair and wool. Its concentration in the other classes of proteins is low and the absorption of ultra-violet rays would be of the same order as that caused by phenylalanine.

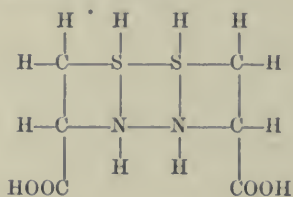
Only the ultra-violet light of the shorter wave lengths has great therapeutic value [Newcomer, 1917]. By shorter wave length is meant the rays of 2800 A.U. and shorter. These wave lengths have, however, very low penetrative power owing probably to the absorption by the outer tissues. The high absorption power of tryptophan, tyrosine and phenylalanine for the rays in the region of 2800 and 2900 A.U. throws some light on the effectiveness of these short wave lengths. These amino acids in low concentration have a high power of absorption and bring about a very complete absorption of these wave lengths. Consequently much energy is available for bringing about changes in the cell. If the amount of energy is so great that the chemical changes upset the normal metabolism of the cell, then the organism is destroyed.

The possible ring structure of cystine.

Cystine is soluble in strong mineral acids such as hydrochloric acid. It is insoluble in weak organic acids such as acetic acid. This is also characteristic of certain ring compounds containing an amino nitrogen as for example indole compounds. It is possible to make chloroacetyl derivatives of cystine by means of chloroacetyl chloride [Fisher and Suzuki, 1904], but apparently it is not possible to make acetyl derivatives of cystine by the use of acetyl chloride. Chloroacetic acid is a much stronger acid than acetic acid, being comparable in strength to hydrochloric acid.

The absorption of cystine is of a greater intensity than that of the aliphatic amino acids alanine and glutamic acid and is as strong as that of phenylalanine.

A possible explanation of these facts is that cystine does not possess the ordinary aliphatic structure usually assigned to it but that it possesses a ring structure, possibly of the following type:



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CXIII. THE ABSORPTION SPECTRA OF KYNURENIC ACID AND SOME RELATED QUINOLINE COMPOUNDS.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(Received August 27th, 1923.)

THE absorption spectra of the following compounds were measured in the course of an investigation into the intermediary metabolism of indolepropionic acid [Ward, 1923, 2]. The injection of indolepropionic acid into the animal organism produces a body in the urine which is very unstable towards heat and is very difficult to isolate. It is the precursor of a bright cherry red pigment. The formation of kynurenic acid from tryptophan [Ellinger, 1904] and indolepyruvic acid [Ellinger and Matsuoka, 1920] suggested the possibility that a similar body might be formed from indolepropionic acid. The plan was to measure the absorption of the precursor of the pigment separated as completely as possible from other compounds found in the urine and to compare this absorption spectrum with the absorption spectra of known quinoline derivatives.

EXPERIMENTAL.

The apparatus and method employed were the same as that described by the present writer in a paper on the absorption spectra of some indole compounds [1923, 1].

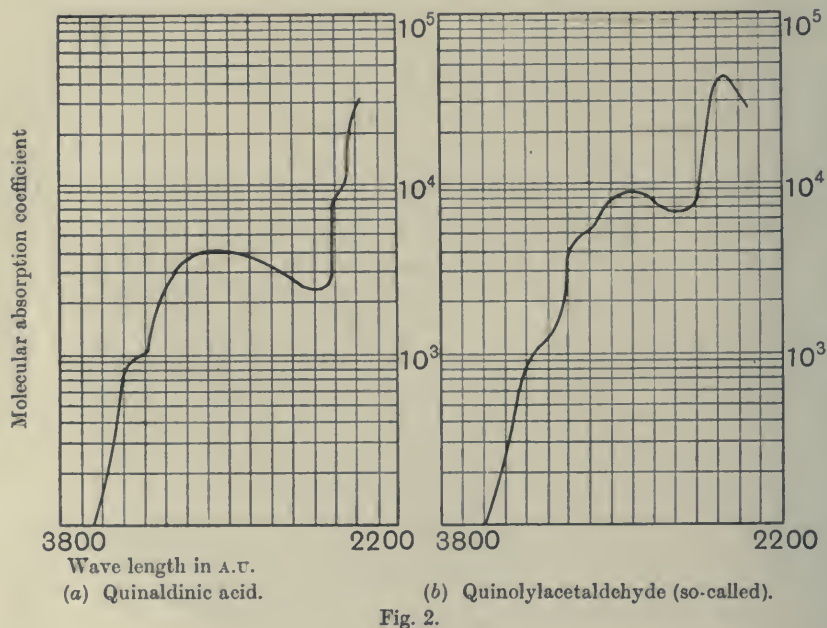
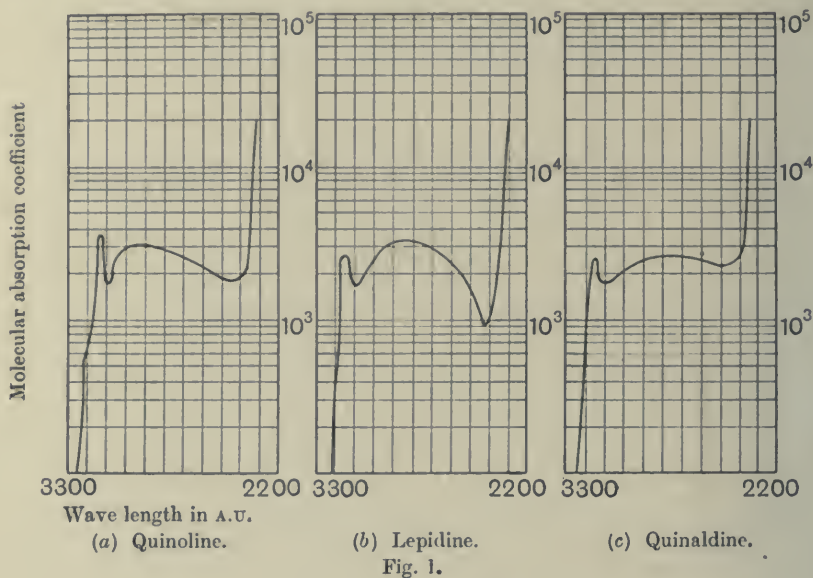
Seven different compounds of the quinoline series were investigated. These were as follows: quinoline, quinaldine, lepidine, quinaldinic acid, quinolyl-acetaldehyde, 2:6-dimethylquinoline and kynurenic acid.

The quinoline and quinaldine were purchased from British Drug Houses. The lepidine, quinaldinic acid, quinolylacetaldehyde and 2:6-dimethylquinoline were obtained from various sources in very small quantities of about two-tenths of a grain.

The kynurenic acid was prepared by feeding 20 g. of tryptophan to a dog at the rate of 4 g. per day. The kynurenic acid was first isolated in a crude state by acidifying the urine with hydrochloric acid and allowing to stand overnight. After filtering off the precipitated crude kynurenic acid and washing with water, it was dissolved in dilute ammonia and reprecipitated with dilute hydrochloric acid. By repeating this several times some of the urinary pigments were removed. It was then boiled up with 95 % alcohol and allowed to

cool and then filtered. This removed some more of the pigment. It was finally recrystallised several times from 40 % acetic acid [Homer, 1914]. The final traces of pigment were removed by boiling in 40 % acid solution with animal charcoal before a final recrystallisation. This gave a white crystalline product.

The results are given in Figs. 1-3.



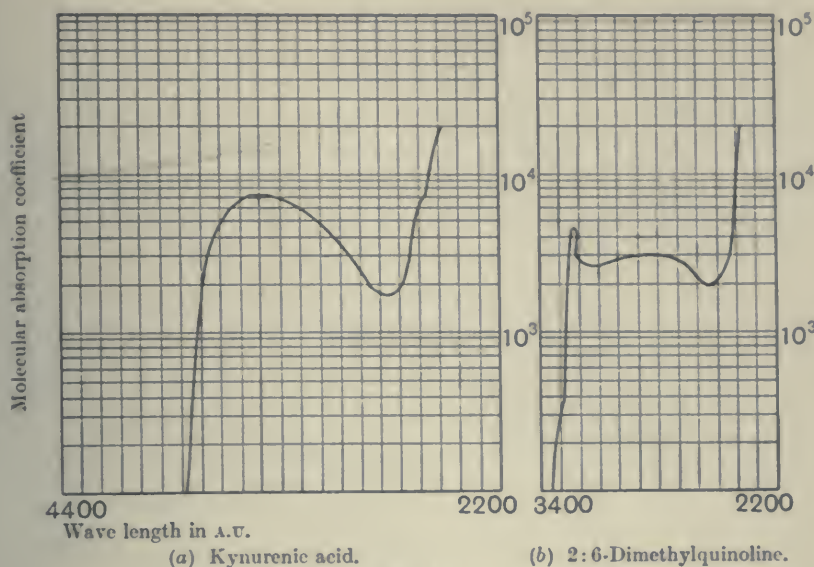


Fig. 3

DISCUSSION.

From the absorption spectra of the three compounds quinoline, lepidine and quinaldine may be observed the difference of effect in the absorption spectra produced in one case by substituting in the α -position and in the other case in the γ -position. Substitution in the α -position produces a shift towards the red end of the spectrum without any increase in the intensity of the absorption. Substitution in the γ -position produces an increase in intensity of the absorption without producing any shift of the absorption band towards the red end of the spectrum.

Quinaldinic acid is quinoline substituted in the α -position by a carboxyl group. This brings about a well-marked change in the absorption as compared with quinaldine. There is a shift towards the red end of the spectrum of about 400 A.U. and there is also an increase in intensity.

Quinolylacetaldehyde is the name under which the compound whose absorption spectrum is given in Fig. 2 *b* is described in the literature [Einhorn, 1885, 1886; Carlier and Einhorn, 1890; Einhorn and Sherman, 1895]. It gives the correct elementary analysis for this constitution but it does not behave in its reactions as such a compound. It gives a hydrazone with great difficulty and in very poor yield. The absorption spectrum is not such as would be expected of such a compound.

2:6-Dimethylquinoline is a compound with a methyl group substituted in both rings. It retains a strong resemblance to quinaldine and quinoline. The effect of the double substitution is to increase the intensity of the absorption and to shift the absorption band towards the red end of the spectrum.

In kynurenic acid there is substitution in the α -position by a carboxyl group and in the γ -position by a hydroxyl group [Homer, 1913]. The presence of the carboxyl group in the α -position brings about a shift towards the red end of the spectrum with an increase in intensity. The presence of the hydroxyl in the γ -position brings about an increase in intensity. The effect of the two substitutions is to give a big shift towards the red end of the spectrum and greatly to intensify the absorption.

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CXIV. THE FATE OF INDOLEPROPIONIC ACID IN THE ANIMAL ORGANISM.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(Received August 27th, 1923.)

INDOLEPROPIONIC acid was first prepared by Nencki [1889] by the action of anaerobic bacteria upon commercial fibrin. He used three different strains of bacteria, namely *B. liquefaciens magnus*, *B. spinosus* and the Rauschbrand bacillus. He obtained the best yield of indolepropionic acid by the use of the Rauschbrand bacillus.

Hopkins and Cole [1903] subjected tryptophan to bacterial decomposition and obtained indole, indoleacetic acid and indolepropionic acid, proving that the amino acid was the precursor of the putrefactive decomposition bodies, indole, skatole, indoleacetic acid and indolepropionic acid. Indolepropionic acid was prepared by these workers by means of the Rauschbrand bacillus and *B. coli* working under anaerobic conditions in an atmosphere of hydrogen or nitrogen.

Ellinger [1904] fed tryptophan to a dog and found that there was an increased output of kynurenic acid in the urine. Some confusion existed for a long time as to the correct constitution of kynurenic acid, the two alternative forms being γ -hydroxy- α -carboxyquinoline and γ -hydroxy- β -carboxyquinoline. Homer [1913, 1914], by carefully recrystallising crude kynurenic acid from 40 % acetic acid, found that the melting-point agreed with that of γ -hydroxy- α -carboxyquinoline as prepared by Camps [1901].

Homer [1915] fed kynurenic acid to a dog but the material was excreted unchanged by the organism. It appears in that animal to be an end product of metabolism and not some intermediate product that has been excreted through being in excess.

Ellinger and Matsuoka [1914] prepared α -methyltryptophan and fed the product to a rabbit. They expected to obtain a methyl derivative of quinoline and to settle thereby in which form kynurenic acid exists. The only result of this experiment was that the α -methyltryptophan was recovered unchanged in the urine.

Barger and Ewins [1917] prepared α -methyltryptophan and fed it to a small dog. No kynurenic acid was found in the urine, the material being excreted in a form that left the indole ring intact.

Ellinger and Matsuoka [1920] prepared indolepyruvic acid and injected it into a rabbit. They obtained kynurenic acid in varying yields up to 11.7 %.

Injections of tryptophan gave yields of kynurenic acid up to 28 %. They also fed quinaldinic acid to rabbits but failed to obtain kynurenic acid as the oxidation product and found instead the quinaldinic acid conjugated with glycine.

Recently Matsuoka and Takemura [1922] have perfused the surviving liver of dogs and claim to have isolated kynurenic acid to the extent of 12 % after the addition of tryptophan and indolepyruvic acid to the perfusing fluid.

Homer [1915] made some spectrographic examinations of the pigment formed from indole compounds and from the urine of dogs fed with various indole compounds. She concluded that indole, skatole, indole-aldehyde, indole-carboxylic acid and indoleacetic acid undergo little change in the animal body but that indolepropionic acid appears to undergo a more deep-seated change in its passage through the animal organism.

Ewins and Laidlaw [1913] have shown that indole-ethylamine injected in the animal organism is excreted as indoleacetic acid conjugated with glycine. Similarly, when indoleacetic acid is injected into the animal organism, it is also excreted as a conjugate of indoleacetic acid and glycine. They have also shown that when indole-ethylamine is perfused through the surviving liver, the amine is oxidised to indoleacetic acid.

Guggenheim and Loeffler [1916] claim to have isolated indole-ethyl alcohol as a product of the perfusion of indole-ethylamine besides the previously isolated indoleacetic acid.

EXPERIMENTAL.

Preparation of Tryptophan and Indolepropionic acid.

The tryptophan required for the preparation of indolepropionic acid was made by the method of Hopkins and Cole [1901] with Onslow's modification [1921]. The product was finally extracted by butyl alcohol, according to Dakin's method [1918, 1920], a specially designed vacuum apparatus being used, in which the butyl alcohol boiled at about 65°.

Following the previous work of Hopkins and Cole [1903] several attempts were made to repeat the preparation of indolepropionic acid by means of pure cultures of *B. chauvei* obtained from the Lister Institute. Two such attempts failed and, as it was necessary to wait a month for the completion of an experiment, much time was lost. Finally twelve different strains of anaerobes were inoculated into small tubes of the media and kept under anaerobic conditions for a month in an incubator. These small tubes were tested after this lapse of time and four of them were found to have traces of ether-soluble derivatives of indole. These four were *B. coli*, *B. chauvei*, *B. sporogenes* and *B. oedemaciens*. Fresh twenty-four hour cultures were made of these bacteria and a five-litre bottle filled with sterilised medium containing 25 g. of tryptophan was inoculated with the whole four freshly growing cultures. The bottle was stoppered with a cork containing tubes for passing nitrogen gas through the bottle and with a mercury seal on the outlet. The air was removed by passing nitrogen through the culture and medium for a period of five hours.

When the bottle was opened at the end of four weeks, it was found to smell strongly of indole derivatives. In order to obtain the indolepropionic acid the solution was acidified with 5 % by volume of sulphuric acid and the indole compounds precipitated with mercuric sulphate-sulphuric acid solution. The precipitate was allowed to stand for twenty-four hours and then filtered off and washed. It was then suspended in warm water, made alkaline with baryta and the mercury compound decomposed with hydrogen sulphide. The solution was filtered from mercury sulphide and the residue washed. The filtrate and washings were united and acidified and extracted in the extraction apparatus with ether for four or five hours. The ether was then removed and the sticky mass of crystals was dissolved in alcohol, reduced in volume on a water-bath and water added. The indolepropionic acid was recrystallised from dilute alcohol after removing pigment by boiling with animal charcoal.

The average yield was about 30 % of the crude tryptophan used. In all, about 30 g. of indolepropionic acid were prepared.

Metabolism experiments with Indolepropionic acid.

Indolepropionic acid was dissolved in sodium bicarbonate solution and injected subcutaneously into a rabbit and the urine collected. When the urine was acidified with strong hydrochloric acid and boiled it developed a cherry-red colour. This colour is different from that obtained on injecting indoleacetic acid, acidifying the urine with hydrochloric acid, adding a drop of ferric chloride and boiling. It is also different from that obtained on heating free indoleacetic acid with hydrochloric acid.

3 g. were injected in the course of three days into four rabbits and the urine collected, care being taken to keep the collected urine in a cool place in presence of a preservative. The collected samples were precipitated with mercuric sulphate reagent by adding it until no further precipitate was obtained. The precipitate was filtered off and washed with distilled water. It was then suspended in distilled water, made alkaline with baryta, and decomposed with hydrogen sulphide. The mercury sulphide was filtered off and the baryta exactly removed by dilute sulphuric acid. The filtered solution was then evaporated to dryness on the water-bath. A red sticky gum was obtained which was dried in a vacuum desiccator over sulphuric acid. The red coloration developed as the evaporation proceeded and could not be prevented except in alkaline solution. It appeared to be a decomposition product of the precursor in the urine derived from the indolepropionic acid. Once the red pigment was formed, it acted very much like an indicator, turning yellow in alkaline solution and red in acid solution. Several attempts to purify this material by recrystallising were without success. The compound was further purified by dissolving in alkali, acidifying and extracting with butyl alcohol. It was then removed from the butyl alcohol by shaking with alkaline solutions of baryta, the baryta removed with dilute sulphuric acid and the solution again evaporated to dryness. The material was still tarry and could not be

got to crystallise from any of the ordinary solvents. This procedure was repeated several times but was not attended with any greater success.

Perfusion experiments were carried out with indolepropionic acid, tryptophan and indole-ethyl alcohol, but the only products that could be isolated were tarry materials soluble in alcohol but very resistant to all attempts at purification by recrystallisation. The liquid obtained at the end of a perfusion of tryptophan gave a positive nitroprusside test, as given by acetoacetic acid.

Absorption Spectra Experiments.

Owing to the difficulty of obtaining satisfactory results by the usual methods, it was evident that some other procedure must be used to obtain information as to the type of compound formed in the metabolism of indolepropionic acid. It was decided to attempt to make use of the quartz ultra-violet spectrophotometer in order to find out whether it would throw any light on the constitution of the compound present in the urine. There was the further inducement that it would be possible to record the absorption spectra of the compound without having to apply heat in its isolation.

In order to make use of this method it was first necessary to consider the possible metabolic products that might be obtained. This could be done by analogy with the known metabolism of similar compounds. Having listed the possible end products of the metabolism of indolepropionic acid, it was then necessary to make a study of these and related compounds as far as obtainable in order to observe the general shape of the absorption spectra curves and the effect of changing the substituting groups.

There appear to be four possible courses that the metabolism of indolepropionic acid could take which are as follows:

I. β -Oxidation of the side chain giving indolecarboxylic acid.

II. The oxidation of the carbon atom in the pyrrole ring giving rise to α -enol or α -keto indole derivatives.

III. A combination of types I and II giving rise to α -enol or α -keto β -carboxyindole.

IV. A splitting of the pyrrole ring and a linking up again with the formation of a quinoline derivative.

Following out this plan the absorption spectra of ten indole and seven quinoline compounds were measured. These divide themselves naturally into three groups.

Group I. *β -Substituted indole derivatives.*

(a) Tryptophan, indolepropionic acid and indole-ethyl alcohol.

(b) Indole, β -indolecarboxylic acid and β -indole-aldehyde.

Group II. *α - and β -Substituted indole derivatives.*

(a) $\alpha\beta$ -Dihydroxyindole and α -hydroxy- β -indole-aldehyde.

(b) Isatin and sodium indigosulphonate.

Group III. *Quinoline compounds.*

Quinoline, quinaldine, lepidine, quinaldinic acid, quinolylacetaldehyde, 2:6-dimethylquinoline and kynurenic acid.

The spectra of the compounds of groups I and II have been described in a paper on indole compounds [1923, 1], and those of group III in a paper on kynurenic acid and some related quinoline compounds [1923, 2].

The absorption Spectra of the Metabolic Product of Indolepropionic acid.

Indolepropionic acid was injected subcutaneously into four rabbits and the urine collected over a period of twenty-four hours. The amount of indolepropionic acid used was 1 g. The urine was treated as described on p. 909, but the filtrate from the mercury sulphide was acidified and extracted with butyl alcohol. The butyl alcohol was then extracted with weak caustic soda solution and the separated aqueous layer was acidified and re-extracted with butyl alcohol. It was again extracted with dilute alkali and the alkaline solution acidified. This time the aqueous solution was extracted with ether and the ether extract washed with water. The aqueous solution was afterwards again extracted with butyl alcohol. In this manner two fractions were obtained, one soluble in butyl alcohol alone and the other soluble in butyl alcohol and in ether.

From these two fractions dilutions were made and the absorption spectra of the material measured. In these absorption spectra measurements there are two difficulties to be met with. The first of these is that the solutions are not pure and the second is that the concentration of the material in the solutions is unknown making it difficult to plot the results. However, it is possible to avoid these troubles to a certain extent. If the material to be determined is present in the solution in considerably greater concentration than the impurities, then these impurities will only interfere in the higher concentrations. As for the concentration certain corrections can be introduced based on the position of the peak of the bands and where they should appear in the correct concentrations.

The absorption spectra for indolepropionic acid and its metabolic product are given in Fig. 1. Fig. 1 *b* gives the absorption spectra of the ether-soluble fraction plotted in two ways, the lower uncorrected and the upper corrected for concentration. Fig. 1 *c* gives the absorption spectra curves of the alcohol-soluble fraction, the lower curve being uncorrected and the upper one corrected for concentration.

In order to show that this type of curve was due to some metabolic product of indolepropionic acid and not to impurities from the urine, two other compounds were also studied in the same way. These were indole-ethyl alcohol and indolecarboxylic acid.

Indole-ethyl alcohol. 1 g. was dissolved in warm water and injected subcutaneously into four rabbits and the urine collected over a period of twenty-four hours. The urine was treated in exactly the same way as in the case of indolepropionic acid and the absorption spectra curves obtained of the two fractions. Further the ether-soluble fraction was identified as containing free indoleacetic acid. This was done in the following way. The ether solution was

treated with light petroleum to precipitate the pigments and tarry material. It was then allowed to stand and the clear solution decanted from the precipitated material. The mixed ethers were then evaporated on the water-bath. White waxy crystals were obtained which melted at 164° . The melting point of indoleacetic acid is given as 165° . Some of the material was dissolved in

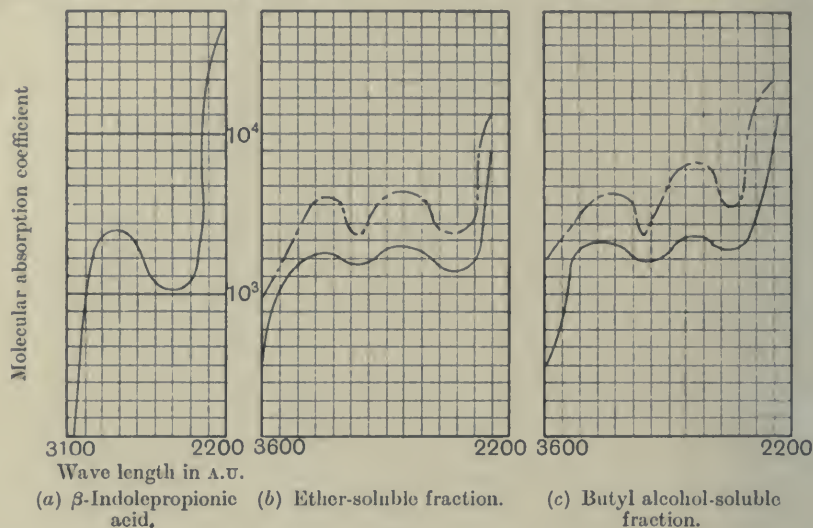


Fig. 1.

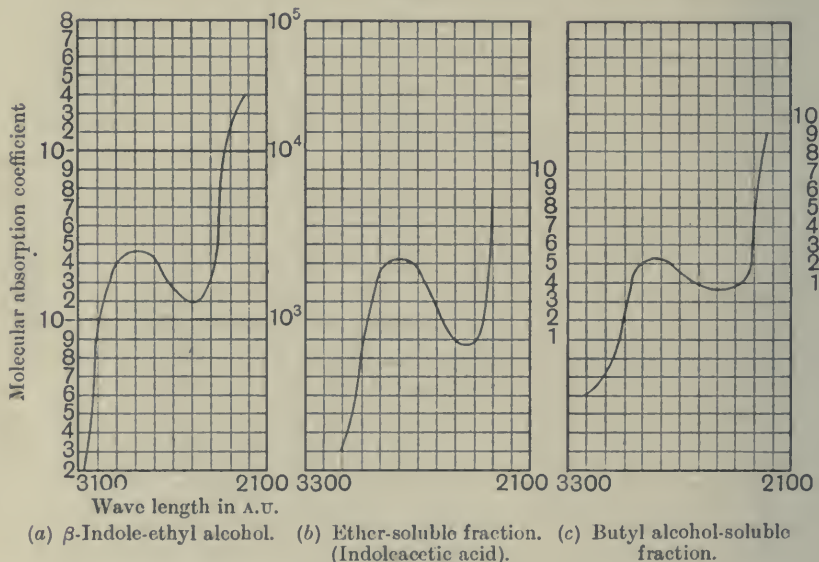
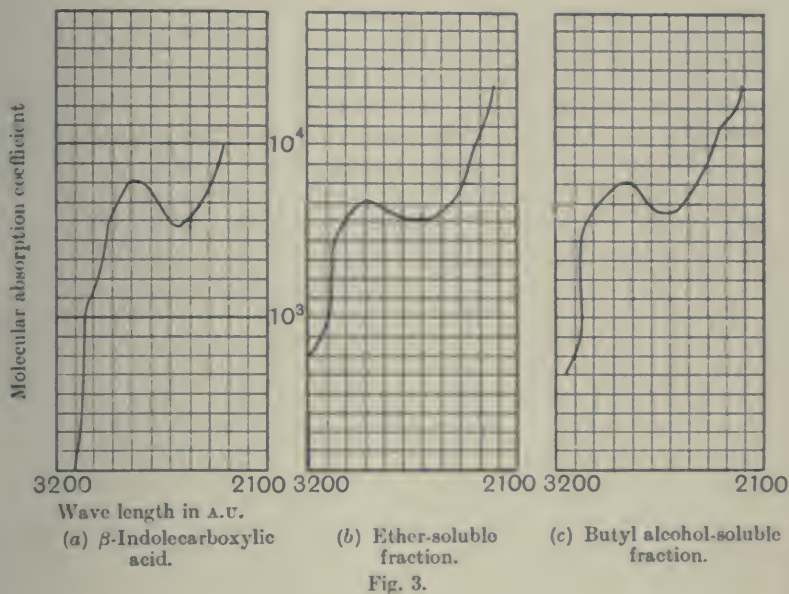


Fig. 2.

water in a test-tube and boiled with strong hydrochloric acid. A red pigment was formed as given by free indoleacetic acid. The original urine also gave a red colour on boiling with strong hydrochloric acid and a drop of ferric chloride solution. The absorption spectra curves are shown in Fig. 2.

Indolecarboxylic acid. About a gram was injected into four rabbits and the urine collected over a period of twenty-four hours. The urine was treated as previously described for indolepropionic acid. Two fractions were again obtained and the absorption spectra measured. The urine on heating with hydrochloric acid gave a purplish colour but different from that given by indolepropionic acid. The absorption curves are given in Fig. 3.



DISCUSSION.

On examining the curves given for indole-ethyl alcohol and for indolecarboxylic acid, it can be seen that there has been no marked change in the absorption spectra given by the metabolic products from that given by the original substance. As can be seen by comparing them with the absorption spectra of β -substituted indole compounds they still retain the shape common to these.

The absorption spectra of indolepropionic acid metabolic products show a marked difference from those of the original substance. The absorption spectra of these substances contain two wide bands, which is not characteristic of β -indole substituted compounds. On comparing the spectra with those given by quinoline compounds, no resemblance can be traced. However, on comparing the absorption spectra of the $\alpha\beta$ -substituted compounds there is a noticeable resemblance.

In Fig. 4 the known absorption spectra curves of three β -substituted indole compounds and the absorption curves of two $\alpha\beta$ -substituted compounds have been plotted. From these curves the absorption spectra curve of α -hydroxy- β -indolecarboxylic acid has been deduced and plotted. The resemblance

between the curve thus deduced and the curves of the metabolic products of indolepropionic acid is evident and strongly favours the suggestion of the formation of an α -hydroxyindole compound as the metabolic product of indolepropionic acid.

Attempts were made to prepare this compound from α -hydroxy- β -indole-aldehyde by oxidation but it appeared to be very unstable as would be expected from the experience with the urinary compound. Several different oxidising reagents were employed but without success. It would appear necessary to stabilise the ring by methylating the hydroxyl group.

The fact that tryptophan and indolepyruvic acid yield kynurenic acid on being injected into the animal organism and the fact that α -methyltryptophan is excreted unchanged, suggest the possibility that the condition of the

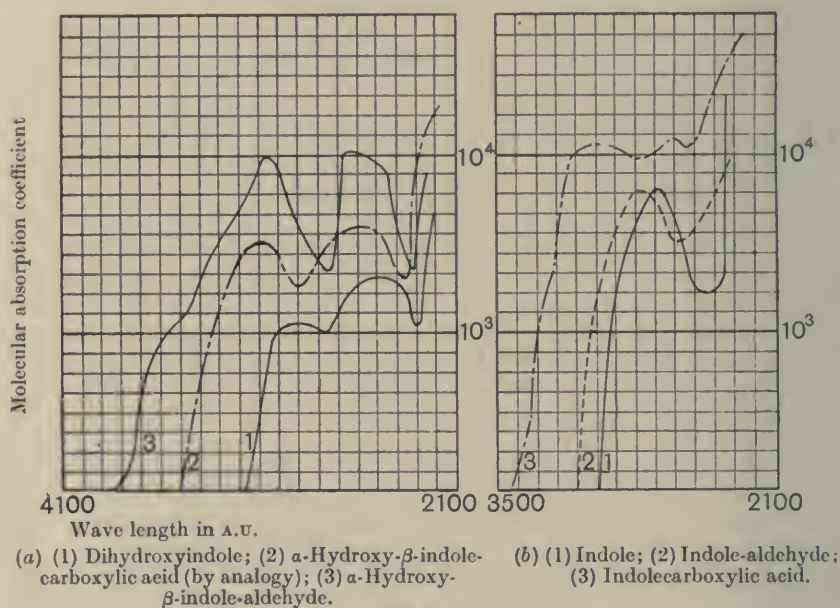


Fig. 4.

α -carbon atom in the pyrrole ring plays an important part in the metabolism of the indole ring as present in tryptophan. The presence of a methyl group would prevent oxidation at this point.

That the α -carbon atom is chemically reactive is shown from other sources. Hopkins and Cole [1903] oxidised tryptophan with ferric chloride and obtained two bases one of which was shown by Ellinger [1904] to be indole-aldehyde and the other was shown by Perkin and Robinson [1919] to be identical with harman, a derivative of harmine. Perkin and Robinson have prepared this substance by oxidising tryptophan in the presence of acetaldehyde. Under this treatment the end of the aliphatic side chain joined on to the α -carbon atom.

SUMMARY.

Indolepropionic acid in its passage through the animal body appears to undergo oxidation in the α -carbon atom of the pyrrole ring with formation of an α -hydroxyindole compound, which may be in either the enol or keto form.

Indole-ethyl alcohol in its passage through the animal organism undergoes oxidation to indoleacetic acid.

Indolecarboxylic acid, in its passage through the animal body, appears to be unchanged except that it is excreted in a conjugated form. It is not an intermediate step in the metabolism of indolepropionic acid.

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CXV. COLLOIDS AND HAEMOLYSIS.

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IN the investigation of haemolytic phenomena the majority of experimenters have made use of washed corpuscles, which are more or less completely divested of the colloids of the plasma. During life, however, the erythrocytes of the blood are invariably associated with colloidal solutions. If, then, we are to gain any insight into the nature of haemolysis in the living organism, it appears essential to study the action of haemolytic substances also in association with colloids.

That serum exerts a protective action in haemolysis has been demonstrated by a number of observers. Moore, Wilson and Hutchinson [1909], Kerner [1910] and Sutherland and Mitra [1916] all recorded the inhibitory action of serum on haemolysis by sodium oleate, while Barker [1922] has noted the differences in the inhibitory power against haemolysis by this substance afforded by sera obtained from human blood during various forms of anaemia. Kuno [1918] found that the lysis produced by heating human erythrocytes was inhibited by the presence of human serum. Fenn [1922] stated that 0.1 % of serum inhibits the haemolysis arising from the contact of erythrocytes with soiled glass. Ponder [1921, 1922, 1923] observed that serum inhibits the haemolytic action of urine and affords protection to human erythrocytes against haemolysis by a histamine-glycocholate mixture, by sodium taurocholate, by sodium glycocholate and by saponin.

Schmidt and Norman [1920] described the inhibition of the haemolysis produced by eosin in the presence of sunlight by serum, egg-white, caseinogen, edestin and "peptone" and stated that gelatin failed to restrain this photodynamic action. Pickering and Collins [1923] pointed out the protective action of isotonic gum-saline against the haemolysis of human erythrocytes at 13° by X-rays.

The objects of the present investigation are as follows: to ascertain the variations in the resistance to haemolysis of erythrocytes obtained from different individuals of the same species, both human and animal, and the effect of storage thereon: to enquire into the influence on haemolysis of various colloids, viz. gum acacia, gelatin, egg-white and sera, fresh and "inactivated": to investigate the influence of varying concentrations of saponin and of a naturally haemolytic serum on the speed and titre of haemolysis in the presence

and absence of the colloids named and to enquire into possible modifications in the protection afforded by these colloids by alterations in the hydron concentration in the media employed.

Eisler [1909] found that sugar inhibits specific haemolysis, that the addition of moderate amounts of various salts restores lysis, higher concentrations of the electrolytes again inducing inhibition. Purdy and Walbum [1922] found that certain metallic salts hasten haemolysis by saponin at some concentrations and show opposite effects at other concentrations. Periodicity or a so-called zone phenomenon has been described by Buchanan [1919] in the agglutination of meningococci by cerous nitrate, while Holker [1923] has demonstrated the occurrence of periodicity in the influence of increasing concentrations of sodium chloride on the opacity of human serum. Early in this investigation the influence of saponin dissolved in isotonic saline on haemolysis was found to be often periodic in nature. The effect of colloidal solutions on this periodicity was therefore studied.

The Technique and Terms Employed.

The tests for haemolysis were carried out in Dreyer's tubes both in the water-bath at 38°, controlled by a thermostat, and at room temperatures, which were read and noted at the time of each experiment. Haemolysis in the water-bath took place in the absence of light (except at the actual times of reading). The experiments at room temperatures were in the presence of diffuse daylight. The possible influence of daylight and of ultra-violet light on haemolysis by saponin and by the heterologous serum employed will be enquired into later. By "commencement" of haemolysis we mean the first change in opacity visible to the naked eye by transmitted light, by "completion" when the contents of the tube were free from residue, when similarly observed. In the tables "H. begins" indicates the time of "commencement" of haemolysis; "H. ends" shows the time of "completion" of that process.

The blood corpuscles employed were those of the sheep, guinea-pig, white rat and man. Except in the case of whole blood they were obtained by bleeding the animals into sterile glass tubes, defibrinating with glass beads, thrice washing in normal saline and centrifuging. 25 % suspensions were made up in sterile tubes in 0.86 % NaCl, dissolved in distilled water. The haemolysing agents used were saponin (Merck) and the serum of a rabbit which was haemolytic at 38° to all the erythrocytes used, except those of the rabbit. Variations in the p_H of the protective colloids were made by rendering them neutral, faintly alkaline and faintly acid to litmus, by the addition of small quantities of $N/100$ HCl and $N/100$ NaOH respectively. The protective action in each case was then tested. Control experiments showed that the colloidal substances so treated were devoid of haemolytic action.

In each experiment the bulk of the haemolytic substance together with the protective colloid employed and the normal saline used for dilution was 1 cc. to which 0.004 cc. of a 25 % suspension of the various corpuscles was added.

Complete haemolysis is indicated by +, when more than half of the suspension is haemolysed \pm is used, when less than half \mp is recorded. The letters N.C.H. imply nearly complete haemolysis. Each suspension undergoing haemolysis was compared with a standard suspension showing no haemolysis after centrifuging or settling out.

Variations in the Resistance of Erythrocytes to Haemolysis.

Considerable variations occur in the titre and speeds of haemolysis in individuals of the same species, but erythrocytes withdrawn under the same conditions on consecutive days from the same animal exhibit but little alteration in sensitiveness to haemolysis by saponin and by the haemolytic serum used. With human whole blood individual variations in sensitiveness to saponin are well marked, as shown in Table I. The temperature of haemolysis was 13°. Two readings (marked A and B) of the commencement and completion of haemolysis are shown for each concentration of saponin on corpuscles from bleedings from the same person taken at an interval of one week. In each case the reading B was with blood withdrawn one week later than that used for reading A. Each bleeding was made in the afternoon.

Table I. *Showing Individual Variations in the Sensitiveness of Human Blood to Haemolysis by Saponin.*

Concentrations of saponin in 0.86 % NaCl		1/10,000 A	1/10,000 B	1/20,000 A	1/20,000 B
1. age 22	{ H. begins	2' 0"	2' 10"	3' 30"	3' 30"
♂	{ H. ends	3 30	3 30	10 30	10 15
2. age 23	{ H. begins	2 30	2 30	trace 1 hr.	- 1 hr.
♂	{ H. ends	6 0	5 55	+ 24 hrs.	+ 24 hrs.
3. age 26	{ H. begins	2 30	2 25	\pm 1 hr.	\pm 1 hr.
♂	{ H. ends	3 20	3 30	+ 24 hrs.	+ 24 hrs.
4. age 29	{ H. begins	1 10	2 0	- 1 hr.	- 1 hr.
♂	{ H. ends	4 0	4 0	trace 24 hrs.	trace 24 hrs.
5. age 30	{ H. begins	0 55	1 0	- 1 hr.	trace 1 hr.
♂	{ H. ends	1 30	1 35	\pm 24 hrs.	\pm 24 hrs.
6. age 31	{ H. begins	1 40	1 45	- 1 hr.	- 1 hr.
♂	{ H. ends	3 0	3 0	N.C.H. 24 hrs.	N.C.H. 24 hrs.
7. age 55	{ H. begins	1 0	1 0	- 1 hr.	- 1 hr.
♂	{ H. ends	4 0	3 35	\pm 24 hrs.	\pm 24 hrs.
8. age 26	{ H. begins	3 30	3 30	- 1 hr.	- 24 hrs.
♀	{ H. ends	4 0	4 5	trace 24 hrs.	
9. age 28	{ H. begins	2 0	2 10	- 1 hr.	\mp 1 hr.
♀	{ H. ends	4 30	4 30	trace 24 hrs.	\mp 24 hrs.
10. age 35	{ H. begins	2 30	2 30	- 1 hr.	- 1 hr.
♀	{ H. ends	4 0	4 10	trace 24 hrs.	\mp 24 hrs.
11. age 48	{ H. begins	1 0	1 10	- 1 hr.	- 24 hrs.
♀	{ H. ends	1 30	1 30	trace 24 hrs.	
12. age 54	{ H. begins	2 0	2 30	- 1 hr.	- 1 hr.
♀	{ H. ends	3 10	3 10	+ 24 hrs.	\pm 24 hrs.

The following may be noted:

(1) The speeds of haemolysis by saponin of whole blood taken from the same person at the interval of one week are remarkably constant.

(2) The relatively rapid haemolysis of whole blood by a high concentration of saponin does not imply a relatively low resistance to lower concentrations of saponin. Thus the corpuscles of one individual (No. 11) which were completely haemolysed in 1' 30" by 1/10,000 saponin, exhibited incomplete haemolysis after exposure for 24 hours to 1/20,000 saponin, whereas the corpuscles of another individual (No. 1) which were haemolysed in 3' 30" by 1/10,000 saponin, exhibited complete haemolysis after exposure for 10' 30" to 1/20,000 saponin. In an isolated case (blood from a male aged 25), 1/40,000 of saponin gave partial haemolysis in 2 hours and complete haemolysis in 24 hours.

(3) Similar variations in individual sensitiveness to haemolysis by saponin occur with the thrice washed corpuscles of man, the guinea-pig, white rat, sheep and rabbit, greater variations being found in the speed than in the titre of haemolysis of the corpuscles examined; those of the sheep appear to exhibit the least individual variations in sensitiveness to haemolysis by saponin and, in this connection, the high resistance exhibited by the erythrocytes of this animal to haemolysis by saponin may be recalled. The thrice washed corpuscles of the guinea-pig, which are hypersensitive to saponin, exhibit as marked individual variations in sensitiveness to haemolysis by that substance as does the whole blood of that animal or the whole blood or washed erythrocytes of man.

It was found that the resistance of sheep's corpuscles to saponin, as shown by the titre and speed of haemolysis, underwent little alteration when the erythrocytes were kept for two days in sterile tubes at room temperatures (13°–14°) or for three days in an ice-chest. After these periods resistance is definitely lower as is shown by the next table.

Table II. *Showing Deterioration in Resistance to Haemolysis - by Saponin due to Storage.*

Concentrations of saponin dissolved in 0.86 % NaCl		1/800	1/1600	1/3200	1/6400	1/12800	1/25600
Fresh erythrocytes (sheep)	{ H. begins	3' 50"	9' 30"	21' 30"			
	{ H. ends	4 40	18 30	39 30	- 24 hrs.	- 24 hrs.	- 24 hrs.
Erythrocytes kept 4 days in ice-chest (sheep)	{ H. begins	2 0	2 30	8 0	12' 0"	18' 0"	- 1 hr.
	{ H. ends	3 0	4 15	14 0	28 0	32 0	± 24 hrs.

A similar lowering of resistance to haemolysis by saponin was observed when the erythrocytes of the guinea-pig, white rat and rabbit were kept, under the conditions named, for similar periods.

Table III. *Showing Deterioration in Resistance to Haemolysis by a Heterologous Serum due to Storage.*

Percentages of rabbit's serum employed		25 %	12.5 %	6.25 %	3.125 %
Fresh erythrocytes (sheep)	{ H. begins	2' 0"	16' 0"	- 2 hrs.	- 24 hrs.
	{ H. ends	4 0	18 0	- 24 hrs.	
Erythrocytes kept 4 days at 13° (sheep)	{ H. begins	at once	9 0	21' 0"	- 24 hrs.
	{ H. ends	..	15 0	42 0	

Similar results were obtained with fresh and "four day" guinea-pig's corpuscles, when the haemolysing agent was serum from the same rabbit.

The Protective Action of Gum Acacia.

A 7 % solution of gum acacia, dissolved in 0.86 % NaCl, was used. One half of the fluid of the suspensions was of this material, the remainder being saponin, in the dilutions named, dissolved in isotonic saline. In the control experiments the suspensions were in 0.86 % NaCl. The protective action of gum-saline against the haemolysis at 13° by saponin of fresh sheep's corpuscles is usually very slight and may be absent. When the corpuscles have remained in sterile tubes in an ice-chest, excluded from daylight, the protective action of gum acacia is well marked at 13° against certain concentrations of saponin, as is shown in Table IV.

Table IV. *Showing the Protection afforded by Gum-saline against the Haemolysis of Stored Erythrocytes by Saponin.*

Concentrations of saponin used	1/800	1/1600	1/3200	1/12800	1/25600	1/51200
Sheep's erythrocytes suspended in 0.86 % NaCl	{ H. begins 2' 0"	2' 30"	8' 0"	18' 0"	- 1 hr.	- 24 hrs.
	{ H. ends 3 0	4 15	14 0	33 0	± 24 hrs.	trace 48 hrs.
Sheep's erythrocytes suspended in gum-saline	{ H. begins 2 0	5 50	8 0	- 24 hrs.	- 24 hrs.	- 48 hrs.
	{ H. ends 3 0	12 0	17 30			

Table V records the speeds of haemolysis of the fresh erythrocytes of the sheep at 38°, and of the corpuscles of the guinea-pig, white rat and rabbit, at room temperatures, by saponin dissolved in 0.86 % NaCl and by like concentrations of saponin in the presence of 50 % of a 7 % solution of gum acacia dissolved in 0.86 % NaCl. The commercial gum acacia employed was faintly acid to litmus and contained salts of calcium.

Hitherto the appearance of periodicity, as illustrated by changes in the agglutination of erythrocytes, in the opacity of serum and in specific haemolysis, has been associated with variations in the concentration of the electrolytes present. Reference to Table V will show that periodicity occurs in the haemolysis of sheep's corpuscles at 38° and at lower temperatures with the erythrocytes of the guinea-pig and white rat, when the suspensions are made and the saponin is dissolved in normal saline. In these cases the variant is the concentration of the saponin and not the total amount of electrolyte present.

That temperature may also be a factor in periodicity in haemolysis is illustrated by the fact that the phenomenon is frequently absent in the haemolysis of sheep's corpuscles by saponin at 13° but is well marked at 38°.

It appears probable that periodicity in haemolysis, under the conditions named, is due to alterations in the local concentrations of electrolytes at the interfaces of the corpuscles by the varying concentrations of saponin present. If this is so, physical phenomena are involved in the inception of haemolysis by saponin.

With the erythrocytes of a guinea-pig suspended in normal saline the presence of 1/51,200 of saponin caused relatively slow haemolysis accompanied by marked sedimentation. The presence of 1/102,400 of saponin gave more rapid haemolysis and sedimentation was absent. The corpuscles of another guinea-pig exhibited slow haemolysis accompanied by sedimentation when

Table V. *Showing the Action of Gum-saline and the Occurrence of Periodicity in the Haemolysis of Fresh Erythrocytes by Saponin.*

Concentrations of saponin used		1/6400	1/12800	1/25600	1/51200	1/102400
Sheep's erythrocytes in 0.86 % NaCl at 38°	{ H. begins	complete	2' 0"	8' 0"	30' 0"	
	{ H. ends	in 60"	3 0	± 2 hrs.	± 24 hrs.	- 48 hrs.
Sheep's erythrocytes in 50 % gum-saline at 38°	{ H. begins	complete	2 30	21' 0"	120'	
	{ H. ends	in 1' 50"	7 30	± 2 hrs. N.C.H. 24 hrs.	slight 24 hrs.	- 48 hrs.
Guinea-pig's corpuscles in 0.86 % NaCl at 13°	{ H. begins	0' 50"	4 30	7' 30"	6' 30"	12' 45"
	{ H. ends	1 30	8 30	13 0	11 30	15 40
Guinea-pig's corpuscles in 50 % gum-saline at 13°	{ H. begins	0 50	5 50	10 0	10 0	
	{ H. ends	1 30	8 30	- 2 hrs. ± 24 hrs.	- 120' ± 24 hrs.	- 24 hrs.
White rat's corpuscles in 0.86 % NaCl at 13°	{ H. begins	0 30	1 30	1' 30"	3' 55"	27' 0"
	{ H. ends	1 20	2 20	2 30	28 0	± 120 0 + 24 hrs.
White rat's corpuscles in 50 % gum-saline at 13°	{ H. begins	0 30	4 0	12 15	- 48 hrs.	- 24 hrs.
	{ H. ends	1 30	55 0	± 24 hrs.		± 48 hrs.
Rabbit's corpuscles in 0.86 % NaCl at 14°	{ H. begins	} at once	1' 0	2 0	3' 50"	7' 0"
	{ H. ends		1 40	3 0	13 0	14 0
Rabbit's corpuscles in 50 % gum-saline at 14°	{ H. begins	1' 50"	4 30	10 20	± 30' 0"	
	{ H. ends	2 10	5 0	12 30	+ 4 hrs.	- 24 hrs.
Concentrations of saponin used		1/204800	1/409600	1/819200	1/1638400	1/3276800
Sheep's erythrocytes in 0.86 % NaCl at 38°	{ H. begins	2' 30"	2' 0"	54' 0"	- 24 hrs.	- 24 hrs.
	{ H. ends	3 30	3 30	± 24 hrs.		
Sheep's erythrocytes in 50 % gum-saline at 38°	{ H. begins	16 0	3 0	3' 50"	- 120' 0"	trace
	{ H. ends	N.C.H. 24 hrs.	8 0	9 0	trace 24 hrs.	24 hrs.
Guinea-pig's corpuscles in 0.86 % NaCl at 13°	{ H. begins	3' 30"	1 20	4 45	40' 0"	
	{ H. ends	3 55	3 20	8 35	N.C.H. 24 hrs.	± 24 hrs.
Guinea-pig's corpuscles in 50 % gum-saline at 13°	{ H. begins	- 2 hrs.	- 2 hrs.	- 24 hrs.	- 24 hrs.	- 24 hrs.
	{ H. ends	± 24 hrs.	± 24 hrs.			
White rat's corpuscles in 0.86 % NaCl at 13°	{ H. begins	28' 0"	7' 0"	- 60' 0"	- 60' 0"	46' 0"
	{ H. ends	± 24 hrs.	N.C.H. 24 hrs.	± 24 hrs.	± 24 hrs.	± 60 0 + 24 hrs.
White rat's corpuscles in 50 % gum-saline at 13°	{ H. begins	- 24 hrs.	- 24 hrs.	- 48 hrs.	- 48 hrs.	- 48 hrs.
	{ H. ends	+ 48 hrs.	+ 48 hrs.			
Rabbit's corpuscles in 0.86 % NaCl at 14°	{ H. begins	- 48 hrs.	- 48 hrs.	"	"	"
	{ H. ends					
Rabbit's corpuscles in 50 % gum-saline at 14°	{ H. begins	"	"	"	"	"
	{ H. ends					

1/102,400 of saponin was present. With 1/204,800 of saponin haemolysis was very rapid and sedimentation was absent. With 1/1,638,400 haemolysis was very slow but sedimentation was absent. Sedimentation of the corpuscles of the guinea-pig was also observed in the presence of gum with concentrations of saponin varying from 1/51,200 to 1/102,400 with corpuscles from different animals, the sedimentation always corresponding with relatively slow haemolysis. With higher titres of saponin sedimentation appeared slight or absent. Sedimentation was not observed when gelatin or egg-white was substituted for gum.

The fresh corpuscles of the sheep at 38°, in the presence of gum-saline, exhibit periodic inhibition of haemolysis by saponin at different concentra-

tions of the latter substance than when the gum is absent. For example, the presence of gum afforded marked protection against 1/25,600 and 1/51,200 of saponin and protection was also afforded against 1/204,800 of saponin, yet with 1/819,200 of that substance haemolysis was slow with the erythrocytes suspended in normal saline but very rapid with those (from the same animal) which were suspended in gum-saline. With 1/102,400 of saponin inhibition of haemolysis occurred with both saline and gum suspensions. A zone phenomenon may be marked in the haemolysis of the corpuscles of the guinea-pig at 13° by saponin dissolved in normal saline, but may be only slightly evident when corpuscles from the same animal are suspended in gum-saline. With the corpuscles of the white rat periodicity in haemolysis by saponin was found to be almost absent in presence of gum (see Table V). In the case of the rabbit it was not found either with protected or unprotected corpuscles, and it was noted that the protection afforded by gum against haemolysis by saponin is greater with the corpuscles of the rabbit than with those of the white rat, guinea-pig or sheep.

That individual variations occur in the appearance of periodicity in the haemolytic action of saponin is shown by Table VI.

Table VI.

Individual Variations in the Periodicity in Haemolysis by Saponin.

Animal type	Temp. of haemolysis	Condition of erythrocytes	Proportion of animals with corpuscles which exhibited periodicity
Sheep	13°	fresh in 0.86 % NaCl	1 out of 5
"	13°	3rd day in 0.86 % NaCl	3 " 5
"	13° and 38°	fresh in gum-saline	each 10 " 10
"	38°	3rd day in gum-saline	14 " 15
Rabbit	13° and 38°	fresh and 3rd day in 0.86 % NaCl	each 0 " 5
"	13° " 38°	" " gum-saline	" 0 " 5
Guinea-pig	13° " 16°	fresh in 0.86 % NaCl	2 " 12
"	38°	" "	4 " 5
"	13°	" gum-saline	1 " 5
"	38°	" "	3 " 5
White rat	13°	" 0.86 % NaCl	3 " 6
"	13°	" gum-saline	0 " 6

Gum saline also protects the fresh erythrocytes of the guinea-pig and sheep against the haemolytic action of fresh rabbit's serum at 38°. (The serum employed was also haemolytic to the corpuscles of the sheep at 38°.)

Table VII. *Showing the Protective Action of Gum-saline against Haemolysis by a Heterologous Serum.*

Proportions of rabbit's serum present		1/2	1/4	1/8	1/16	1/32
Unprotected guinea-pig's corpuscles	{ H. begins } { H. ends }	at once	2' 0" 3 0	3' 30" ± 2 hrs. + 24 hrs.	- 24 hrs.	- 24 hrs.
Guinea-pig's corpuscles protected by gum	{ H. begins } { H. ends }	1' 50" 7 0	56 0 123 0	- 24 hrs.	- 24 "	- 24 "
Unprotected sheep's corpuscles	{ H. begins } { H. ends }	at once	2 0 4 0	6' 30" 8 30	- 2 " ± 24 "	- 2 " ± 24 "
Sheep's corpuscles protected by gum	{ H. begins } { H. ends }	at once	4 0 9 0	120 0 148 0	- 2 " ± 24 "	- 24 " - 24 "

*The Influence on Haemolysis of Alteration in the Hydrion
Concentration of Saponin Solutions.*

Isotonic solutions of saponin were prepared which were respectively neutral, faintly alkaline and faintly acid to litmus. Table VIII illustrates the speed and titre of haemolysis of the erythrocytes of the guinea-pig and rabbit when exposed to these solutions at a temperature of 14°.

Table VIII. *Showing the Titre and Speed of Haemolysis by
Saponin in Neutral, faintly Alkaline and Acid Solutions.*

Concentrations of saponin used		1/12800	1/25600	1/51200	1/102400	1/204800	1/409600
Guinea-pig's corpuscles in neutral saponin	{ H. begins	2' 0"	2' 30"	11' 0"		22' 0"	
	{ H. ends	5 30	5 30	57 0	- 2 hrs.	± 2 hrs.	- 2 hrs.
Guinea-pig's corpuscles in alkaline saponin	{ H. begins	{ at once	0 30	2 0	16' 30"	22' 0"	
	{ H. ends		0 50	5 0	± 2 hrs.		"
Guinea-pig's corpuscles in acid saponin	{ H. begins	{ at once	0 30	2 10	3' 40"	11 0	
	{ H. ends		0 55	3 45	6 50	± 2 hrs	"
Guinea-pig's corpuscles in neutral saponin	{ H. begins	{ at once	0' 40"	2 40			
	{ H. ends		1 30	7 50	18 0	- 2 hrs.	- 2 hrs.
Guinea-pig's corpuscles in alkaline saponin	{ H. begins	{ at once	0 10	0 40	1 10	8' 30"	"
	{ H. ends		0 30	1 50	2 10	24 30	"
Guinea-pig's corpuscles in acid saponin	{ H. begins	{ at once	0 30	0 40	2 50		
	{ H. ends		0 45	1 30	4 50	- 2 hrs.	"
Rabbit's corpuscles in neutral saponin	{ H. begins	{ at once	0 50	1 50	2 10	7' 0"	"
	{ H. ends		1 10	3 10	6 50	20 0	"
Rabbit's corpuscles in alkaline saponin	{ H. begins	{ at once	0 10	0 35	4 40	20' 30"	30' 0"
	{ H. ends		0 40	1 0	17 40	49 0	± 2 hrs.
Rabbit's corpuscles in acid saponin	{ H. begins	{ at once	0' 40"	1 10	1 30	5 50	
	{ H. ends		0 45	1 40	2 20	18 0	- 2 hrs.

Table VIII shows that faintly alkaline and faintly acid saponin are more haemolytic at 14° than is neutral saponin. Like results were obtained when the temperature was 38°. Periodicity in haemolysis by saponin was not observed when the saponin was either faintly alkaline or faintly acid to litmus, even with corpuscles which exhibited periodicity in haemolysis by neutral saponin.

*The Effects of Altering the Hydrion Concentration of the
Protective Colloids.*

Table IX shows the variations in the protection afforded by isotonic gelatin against the haemolysis of fresh erythrocytes of the guinea-pig by saponin at 14° when the added gelatin is made neutral, faintly alkaline and faintly acid to litmus by the addition of hydrochloric acid and sodium hydroxide respectively.

The substitution of gum-saline for isotonic gelatin yields similar results; the gum which is faintly acid to litmus being more protective than gum which is faintly alkaline or neutral.

Table IX. *Showing the Protection against Saponin afforded by Neutral, Alkaline and Acid Gelatin.*

Concentrations of saponin used		1/12800	1/25600	1/51200	1/102400	1/204800	1/409600	1/819200	1/1638400
Unprotected erythrocytes	{ H. begins	1' 50"	4' 50"	6' 40"	53' 0"	2' 30"	1' 30"	5' 45"	40' 0"
	{ H. ends	2 55	12 30	12 10	100 0	3 10	2 30	9 10	± 2 hrs.
Erythrocytes in 50 % neutral gelatin	{ H. begins	3 0	32 0	68 0	96 0	- 48 hrs.	- 48 hrs.	± 24 hrs.	- 48 hrs.
	{ H. ends	7 0	47 0	110 0	122 0			+ 48 hrs.	
Erythrocytes in 50 % alkaline gelatin	{ H. begins	2 40	18 0	30 0	66 0			- 48 hrs.	
	{ H. ends	7 10	36 0	47 0	120 0	"	"		"
Erythrocytes in 50 % acid gelatin	{ H. begins	1 50	8 0	- 24 hrs.	- 48 hrs.	"	"	"	"
	{ H. ends	5 0	± 2 hrs. + 24 hrs.	± 48 hrs.					

In Table X is shown the protective power of neutral alkaline and acid gum against haemolysis of guinea-pig's erythrocytes by rabbit's serum at 38°.

Table X. *Showing the Protection against Haemolysis by a Heterologous Serum afforded by Neutral, Alkaline and Acid Gum.*

Proportions of rabbit's serum employed		1/2	1/4	1/8	1/16	1/32
Unprotected erythrocytes	{ H. begins	at once	1' 30"	2' 50"	30' 0"	- 2 hrs.
	{ H. ends	"	4 20	26 0	± 2 hrs. + 4 "	+ 4 "
Erythrocytes in 50 % neutral gum	{ H. begins	1' 30"	16 0	50 0	100' 0"	- 24 "
	{ H. ends	6 0	32 0	± 2 hrs. + 24 "	± 24 hrs.	
Erythrocytes in 50 % alkaline gum	{ H. begins	4 30	18 0	60' 0"	116' 30"	- 24 "
	{ H. ends	26 0	34 0	± 2 hrs. + 24 "	± 24 hrs.	
Erythrocytes in 50 % acid gum	{ H. begins	1 50	56 0	- 24 "	- 24 "	- 24 "
	{ H. ends	7 0	123 0			

Table XI illustrates the variations in the protective power of 50 % of egg-white, when untreated by reagents and respectively faintly alkaline and faintly acid to litmus, against the haemolysis of the corpuscles of the guinea-pig by saponin at 14°.

Table XI. *Showing the Protection afforded against Saponin by Natural, Alkaline and Acid Egg-white.*

Concentrations of saponin used		1/6400	1/12800	1/25600	1/51200	1/102400
Unprotected erythrocytes	{ H. begins	0' 50"	2' 40"	4' 30"	8' 30"	2' 45"
	{ H. ends	1 30	4 30	7 30	12 50	5 45
Erythrocytes in untreated egg-white	{ H. begins	0 50	2 30	60 0	- 24 hrs.	- 24 hrs.
	{ H. ends	1 30	4 50	± 24 hrs.		
Erythrocytes in alkaline egg-white	{ H. begins	1 0	2 50	- 24 hrs.	"	"
	{ H. ends	1 30	5 0			
Erythrocytes in acid egg-white	{ H. begins	at once	1 0	2' 30"	6' 0"	- 24 hrs.
	{ H. ends		1 30	8 0	60 0	
Concentrations of saponin used		1/204800	1/409600	1/819200	1/1638400	
Unprotected erythrocytes	{ H. begins	2' 35"	1' 20"	4' 45"	40' 0"	
	{ H. ends	3 0	2 20	8 30	N.C.H. 24 hrs.	
Erythrocytes in untreated egg-white	{ H. begins	- 24 hrs.	- 24 hrs.	- 24 hrs.	- 24 hrs.	
	{ H. ends					
Erythrocytes in alkaline egg-white	{ H. begins	"	"	"	"	
	{ H. ends					
Erythrocytes in acid egg-white	{ H. begins	"	"	"	"	
	{ H. ends					

It will be noted that the protection afforded against haemolysis by saponin is greatest when the egg-white is in faintly alkaline solution and is least in faintly acid solution, untreated solutions occupying an intermediate position. The protection afforded by acid egg-white is however considerable. Periodicity in the action of the saponin was exhibited by the unprotected corpuscles, but appeared absent when egg-white was present. The contrast with the behaviour of gelatin in acid and alkaline solutions should be noted (*vide* Table IX). In this connection, it is noteworthy that Bayliss [1906] found that egg-white protects Congo red against the action of electrolytes in alkaline solution but exerts an opposite effect in acid solution.

When the haemolytic agency is the serum of the rabbit (at 38°) and the protective colloid is 50 % of egg-white the results with guinea-pig's corpuscles are different.

Table XII. *Showing Protection by Natural, Alkaline and Acid Egg-white against Haemolysis by a Heterologous Serum.*

Proportions of rabbit's serum used		1/2	1/4	1/8	1/16
Unprotected erythrocytes	{ H. begins	at once	1' 30"	1' 50"	30' 0"
	{ H. ends	"	4 20	26 0	± 120 0 + 24 hrs.
Erythrocytes in natural egg-white	{ H. begins	12' 0"	- 1 hr.	- 1 hr.	- 1 hr.
	{ H. ends	60 0	N.C.H. 24 hrs.	± 24 hrs.	± 24 hrs.
Erythrocytes in alkaline egg-white	{ H. begins	1 10	4' 50"	7' 0"	± 1 hr.
	{ H. ends	6 0	32 0	49 30	± 24 hrs.
Erythrocytes in acid egg-white	{ H. begins	1 30	2 55	6 30	± 1 hr.
	{ H. ends	11 0	135 0	90 0	± 24 hrs.

Similar results to those recorded in Tables XI and XII were found when white rat's corpuscles were substituted for those of the guinea-pig. The erythrocytes of the rabbit exposed to saponin in the presence of acid, alkaline and untreated egg-white and gelatin respectively gave results similar to, but less marked than, those obtained with the corpuscles of the guinea-pig. A serum haemolytic to rabbit's corpuscles was not available.

Table XIII. *Showing Protection by Fresh and "Inactivated" Rabbit's Serum against Haemolysis by Saponin.*

Concentrations of saponin used		1/400	1/800	1/1600	1/3200	1/6400	1/12800
Unprotected sheep's corpuscles	{ H. begins	1' 30"	4' 0"	6' 30"	11' 0"	20' 0"	45' 0"
	{ H. ends	2 30	6 30	9 30	14 10	27 30	± 24 hrs.
Corpuscles protected by fresh serum	{ H. begins	8 0	14 0	- 1 hr.	- 24 hrs.	- 24 hrs.	- 24 hrs.
	{ H. ends	12 0	16 0	N.C.H. 24 hrs.			
Corpuscles protected by serum "inactivated" by storage	{ H. begins	14 0	- 1 hr.	- 24 hrs.	"	"	"
	{ H. ends	48 0	trace 24 hrs.				
Corpuscles protected by serum "inactivated" by heating to 56°	{ H. begins	13 0	trace	"	"	"	"
	{ H. ends	48 0	1 hr. trace 24 hrs.				

The Protective Action of Certain Sera.

Table XIII shows the protection afforded by 50 % of rabbit's serum, when fresh and "inactivated," by heating to 56° and by storage, against the haemo-

lysis of sheep's corpuscles at 11.5° by saponin. The fresh serum employed was haemolytic to sheep's corpuscles at 38°.

Table XIV shows the protection afforded by 50 % of rabbit's serum, when fresh and "inactivated," either by heating to 56° or by storage, against the haemolysis of rabbit's corpuscles at 14° by saponin.

Table XIV. *Showing Protection by Fresh and "Inactivated" Rabbit's Serum against Haemolysis by Saponin.*

Concentrations of saponin used	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400
Unprotected rabbit's erythrocytes	{ H. begins at once H. ends "	at once	1' 0"	1' 5"	2' 50"	6' 40"
Rabbit's erythrocytes in fresh serum	{ H. begins 11' 30" H. ends 14 0	± 24 hrs. + 48 hrs.	- 48 hrs.	- 48 hrs.	- 48 hrs.	- 48 hrs.
Rabbit's erythrocytes in serum "inactivated" by storage	{ H. begins - 48 hrs. H. ends - 48 hrs.	- 48 hrs.	"	"	"	"
Rabbit's erythrocytes in serum "inactivated" by heating	{ H. begins - 24 hrs. H. ends ± 48 hrs.	"	"	"	"	"

The following may be noted concerning the protection afforded by sera against the haemolysis of washed erythrocytes by saponin.

(1) A fresh serum, which is haemolytic at 38°, may be protective at room temperatures (13°-14°) against haemolysis by saponin. Thus a serum may be protective at a low temperature and haemolytic at body temperature.

(2) The sera of the rabbit, guinea-pig and white rat, when "inactivated" either by heating to 56° or by storage, are protective against the haemolysis by saponin of rabbit's, guinea-pig's and white rat's corpuscles at room and body temperatures. The "inactivated" sera exhibit rather greater protective qualities than do the fresh sera.

(3) Rabbit's serum, fresh and "inactivated" either by storage or heating (obtained from an animal haemolytic to the sheep), protects rabbit's corpuscles against haemolysis by saponin at 13° and at 38°. A like protection is afforded by the serum (fresh and "inactivated") of the white rat to the corpuscles of the white rat at 13° and at 38°. Horse serum, when "inactivated" by storage, is powerfully protective against the haemolysis by saponin at 13° and at 38° of the corpuscles of the sheep, guinea-pig and white rat.

Table XV shows the protective power of 50 % horse serum ("inactivated" by storage) against the haemolytic action of fresh rabbit's serum at 38° on the corpuscles of the guinea-pig and the sheep.

Table XV. *Showing Protection by "Inactivated" Horse Serum against Haemolysis by Rabbit's Serum.*

Proportion of rabbit's serum used	1/2	1/4	1/8	1/16
Unprotected corpuscles of guinea-pig	{ H. begins at once H. ends "	2' 0" 3 0	3' 30" ± 24 hrs.	46' 30" ± 24 hrs.
Guinea-pig's corpuscles in horse serum	{ H. begins 2' 0" H. ends 23 0	trace 24 hrs.	- 24 hrs.	- 24 hrs.
Unprotected corpuscles of the sheep	{ H. begins at once H. ends "	2' 0" 4 30	6' 30" 8 30	- 120' 0" ± 24 hrs.
Sheep's corpuscles in horse serum	{ H. begins 60' 0" H. ends N.C.H. 2 hrs. + 24 hrs.	- 24 hrs.	- 24 hrs.	- 24 hrs.

Preliminary experiments with fresh serum of the rabbit and with the "inactivated" serum of the horse indicate that their respective protective qualities are modified by rendering the media of the suspensions faintly acid and faintly alkaline to litmus. It is proposed, however, to defer the publication of this work until more experiments have been conducted.

SUMMARY AND CONCLUSION.

(1) Individual variations occur in the sensitiveness to haemolysis by saponin in whole human blood and in the washed erythrocytes of man, the guinea-pig, white rat, sheep and rabbit.

(2) The individual variations in sensitiveness described are due to variations in the condition of the erythrocytes and not to differences in any plasma or serum which may be adherent to them.

(3) Relatively rapid haemolysis by a high concentration of saponin does not imply a relatively low resistance to lower concentrations of saponin.

(4) The resistance of the erythrocytes of the sheep, guinea-pig, white rat and rabbit to haemolysis by saponin is lowered by keeping the corpuscles in normal saline for longer than three days in sterile tubes at either room temperature or that of an ice-chest.

(5) Periodicity is exhibited in the haemolysis of sheep's corpuscles at 38° by saponin dissolved in 0.86 % NaCl. A similar periodicity occurs in the haemolysis at room temperatures of the corpuscles of the guinea-pig and white rat by saponin dissolved in normal saline.

(6) The occurrence of periodicity in haemolysis by saponin may be modified by alterations in the temperature of the corpuscles.

(7) The presence of gum acacia alters the concentrations at which periodic inhibition of haemolysis occurs in the action of saponin on the erythrocytes of the sheep. Periodicity in haemolysis by saponin appears to be absent when the corpuscles of the white rat and guinea-pig are suspended in gum-saline.

(8) Individual variations occur in the appearance or absence of periodicity in haemolysis by saponin. Periodicity in saponin haemolysis appears to be absent when rabbit's erythrocytes are used.

(9) The association of sedimentation of corpuscles with the exhibition of periodicity in haemolysis is described.

(10) Isotonic gum-saline, isotonic gelatin, egg-white, the "inactivated" serum of the horse, the fresh serum of the rabbit and that "inactivated" either by storage or by heating to 56° protect in varying degrees the erythrocytes of the sheep, white rat, rabbit, and guinea-pig against haemolysis by saponin at room temperatures (13°-14°).

(11) The serum of a rabbit, which at 38° was haemolytic to the corpuscles of the sheep, protects the erythrocytes of the latter animal against haemolysis by saponin at 13° to 14°. The same serum may thus, at different temperatures, be haemolytic and protective respectively.

(12) "Inactivated" serum is more highly protective than is the fresh serum of the same animal.

(13) Gum-saline, isotonic gelatin, egg-white and "inactivated" horse serum protect the erythrocytes of the guinea-pig against the haemolytic action of the serum of the rabbit at body temperature.

(14) The protective action of gum acacia, gelatin, egg-white and of certain sera against haemolysis by saponin or by a haemolytic serum is modified by altering the hydriion concentration of the respective colloids. Faintly acid egg-white is *less* protective against saponin than is faintly alkaline egg-white. Faintly acid egg-white is *more* protective against the haemolysis of guinea-pig's corpuscles by fresh rabbit's serum than is slightly alkaline egg-white.

(15) In view of the results here recorded, it appears premature to accept any of the current hypotheses concerning haemolysis either by saponin or by haemolytic sera. Indications are, however, forthcoming that physical processes play a part in the inception of haemolysis by saponin.

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